INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(72) Inventors; and
(75) Inventors/Applicants (for US only): RAO, Anjana [US/US]; 19 Agassiz Street, Cambridge, MA 02140 (US). HOGAN, Patrick, Gerald [US/US]; 19 Agassiz Street, Cambridge, MA 02140 (US). McCAFFREY, Patricia [US/US]; 35 Ware Road, Newton, MA 02166 (US). JAIN, Jugnu [GB/US]; 21 Eisenhower Avenue, Natic, MA 01760 (US).

(74) Agent: FRASER, Janis, K.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US).

(71) Applicants (for all designated States except US): DANA-FARBER CANCER INSTITUTE, INC. [US/US]; 44 Binney Street, Boston, MA 02115 (US). PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02139 (US).

(54) Title: NF-AT_p, A T LYMPHOCYTE DNA-BINDING PROTEIN

(57) Abstract

    An isolated NF-AT_p protein, a T lymphocyte DNA binding protein, is described. Isolated nucleic acids encoding the protein are also disclosed, as are methods for producing the protein by recombinant means. Methods for screening potential immunosuppressant compounds, which interfere with or inhibit lymphokine gene activation through the NF-AT pathway, are described. Such methods detect phosphorylation of the NF-AT protein.

- 1 -

NF-AT$_p$, A T LYMPHOCYTE DNA-BINDING PROTEIN

Field of the Invention
The invention relates to immunosuppressant
5 compounds.

Background of the Invention
The nuclear factor of activated T cells (NF-AT) is
an inducible, lymphoid-specific transcription factor that
is essential for expression of the IL-2 gene upon T cell
10 activation (For a review, see Ullman, K.S., Northrop,
J.P., Verweij, C.L., Crabtree, G.R. (1990) *Ann. Rev.
Immunol.* **8**, 421-452). By cell fractionation and
reconstitution experiments, NF-AT was shown to be
assembled in the nucleus of activated T cells from a T
15 cell-specific component that is preexisting before
activation and an inducible nuclear component (Flanagan,
W.M., Corthesy, B., Bram, R.J., and Crabtree, G.R. (1991)
Nature **352**, 803-807). The preexisting component of NF-AT
(here designated NF-AT$_p$) was subsequently identified in
20 hypotonic extracts of unstimulated T cells by its ability
to bind specifically to an oligonucleotide corresponding
to the distal NF-AT sequence from the murine IL-2 gene
promoter (Jain, J., McCaffrey, P.G., Valge-Archer, V.E.,
and Rao, A. (1992) *Nature* **356**, 801-804). In addition, it
25 was shown that the inducible nuclear component of NF-AT
consists of Fos and Jun proteins, (Jain et al., *supra*).

- 2 -

Assembly of NF-AT in the nucleus requires two
intracellular signals, the activation of protein kinase C
and an increase in cytosolic free calcium, both of which
are provided by activation of T cells through the T cell
5 antigen receptor. Activation of protein kinase C is
necessary for transcriptional induction of Fos and Jun
genes (Jain, J., Valge-Archer, V.E., and Rao, A. (1992)
J. Immunol. **148**, 1240-1250). An increase in
intracellular calcium is necessary for the appearance of
10 NF-AT$_p$ in the nucleus, presumably reflecting its
translocation from the cytosol (Flanagan, W.M., Corthesy,
B., Bram, R.J., and Crabtree, G.R. (1991) Nature **352**,
803-807). The immunosuppressive drugs cyclosporin A
(CsA) and FK506 block induction of NF-AT by interfering
15 with the calcium-dependent appearance of NF-AT$_p$ in the
nucleus. CsA and FK506, when complexed with their
specific intracellular binding proteins (cyclophilin and
FK506 binding protein, respectively), potently inhibit
the activity of the calcium- and calmodulin-dependent
20 phosphatase, calcineurin (Klee, C.B., Draetta, G.F.,
Hubbard, M.J. (1987) Adv. Enz. **61**, 149-200); Fruman,
D.A., Klee, C.B., Bierer, B.E., Burakoff, S.J. (1992)
Proc. Natl. Acad. Sci. USA **89**, 3686-3690), towards
peptide substrates (Liu, J., Farmer, J.D., Jr., Lane,
25 W.S., Friedman, J., Weissman, I., and Schreiber, S.L.
(1991) Cell **66**, 807-815) Based on these results, it has
been proposed that NF-AT$_p$ in the cytosol may be a target
for calcineurin, either directly as a substrate or
indirectly via a phosphatase cascade (Schreiber, S.L.,
30 and Crabtree, G.R. (1992) Immunology Today **13**, 136-142).

Summary of the Invention

In accordance with the present invention, we have
demonstrated that the DNA-binding component of the NF-AT
T cell nuclear factor, herein referred to as NF-AT$_p$, is a
35 90-140 kDa T cell phosphoprotein that binds directly and

- 3 -

specifically to the NF-AT sequence and that calcineurin
can cause dephosphorylation of this protein in T cell
lysates. These findings directly implicate NF-AT$_p$ in the
chain of events by which CsA and FK506 inhibit IL-2 gene
5 induction.

As described in detail below, NF-AT$_p$ is present in
resting T cells predominantly in a form migrating with an
apparent molecular weight of 110-140 kDa, while NF-AT$_p$
from nuclear extracts of activated T cells migrates with
10 a lower apparent molecular weight (90-125 kDa). This
difference reflects dephosphorylation of NF-AT$_p$, since
treatment of NF-AT$_p$ with calf intestinal phosphatase or
the calcium- and calmodulin-dependent phosphatase
calcineurin *in vitro* results in a similar decrease in its
15 apparent molecular weight. We show that NF-AT$_p$ is
dephosphorylated in cell lysates by a calcium-dependent
process that is blocked by inclusion of EGTA or a
specific peptide inhibitor of calcineurin in the cell
lysis buffer. Moreover, dephosphorylation of NF-AT$_p$ in
20 cell extracts is inhibited by prior treatment of T cells
with the immunosuppressive drugs cyclosporin A (CsA) or
FK506, which inhibit the phosphatase activity of
calcineurin when complexed with their specific binding
proteins, cyclophilin and FK506 binding protein. This
25 work identifies NF-AT$_p$ as a DNA-binding phosphoprotein
and a target for drug/immunophilin/calcineurin complexes
thought to mediate the inhibition of IL-2 gene induction
by CsA and FK506.

In one embodiment, the invention provides a
30 purified preparation of NF-AT$_p$ protein, preferably a
human NF-AT$_p$ protein. The protein can be phosphorylated,
or not phosphorylated. In accordance with the invention,
purified preparations of NF-AT$_p$ protein can be complexed
with a Fos protein, a Jun protein, or with both a Fos and
35 a Jun protein to form a complex resembling the native

- 4 -

nuclear factor of activated T cells (NF-AT), which can be
used to screen for regulatory sequences in the promoter
regions of other cytokine genes or genes of other immune
regulatory proteins for binding to the complexes. Such
5   genes encode proteins that may constitute a family whose
transcription is regulated by a similar mechanism and
provide the basis of drug design strategies to manipulate
the immune response.

In another embodiment of the invention, there is
10 provided an isolated DNA encoding NF-AT$_p$. The DNA
preferably encodes a mammalian NF-AT$_p$ protein or
functional fragment, derivative, or isoform thereof, and
most preferably encodes a human or a murine NF-AT$_p$
protein. The isolated DNA may encode a protein which
15 contains the amino acid sequence of murine NF-AT$_p$ (SEQ ID
NO:5) or human NF-AT$_p$ (SEQ ID NO:12). The invention also
includes isolated DNA containing part or all of either
the sequence of murine NF-AT$_p$ shown in Fig. 22 (SEQ ID
NO:21) or human NF-AT$_p$ shown in Fig. 17 (SEQ ID NO:11),
20 Fig. 21 (SEQ ID NO:19) or Fig. 23 (SEQ ID NO:20). Also
provided are vectors containing the isolated DNA; cells,
which can be prokaryotic or eukaryotic, containing the
isolated DNA; and methods of manufacturing NF-AT$_p$. The
methods comprise culturing the cells containing NF-AT$_p$
25 under conditions permitting expression of the DNA.

In yet another embodiment of the invention, there
are provided antibodies or fragments or variants thereof
that bind to an epitope of the NF-AT$_p$ protein. The
antibodies can be polyclonal or monoclonal, and can
30 recognize an epitope of the NF-AT$_p$ protein in a denatured
or native form. The antibodies, especially the
antibodies that bind to an epitope of native NF-AT$_p$
protein, are particularly useful in detecting expression
of NF-AT$_p$ in a cell. Expression of the NF-AT$_p$ protein can
35 be detected by contacting a mixture of proteins from the

- 5 -

cells of interest with an anti-NF-AT$_p$ antibody of the
invention, which is labeled, and detecting immune complex
formation.

Expression of the NF-AT$_p$ protein in a cell can
5   also be detected by contacting mRNA obtained from the
cell with a labeled hybridization probe comprising, for
example, a single-stranded segment of isolated DNA
encoding a fragment of the NF-AT$_p$ protein and detecting
hybridization of the probe with the mRNA. The invention
10   includes an isolated DNA containing 20 nucleotides that
hybridizes under stringent conditions to a strand of a
DNA encoding NF-AT$_p$. By the term "stringent conditions"
is meant DNA hybridization and wash conditions
characterized by relatively high temperature and low salt
15   concentration, e.g., conditions described in Sambrook et
al., (1989) *Molecular Cloning: a Laboratory Manual*,
second edition., Cold Spring Harbor Press, Cold Spring
Harbor, N.Y), page 7.52 of which is herein incorporated
by reference. The segment of DNA may be 20 nucleotides,
20   preferably 50 nucleotides, more preferably 100
nucleotides, and most preferably 200 nucleotides in
length.

In yet another embodiment, the invention includes
an isolated DNA which encodes a segment of NF-AT$_p$ which
25   binds to Fos-Jun or Jun-Jun, e.g., nucleotides 672-2063
of SEQ ID:21. By "Fos-Jun" is meant the heterodimeric
complex of the transcription factors Fos and Jun. By
"Jun-Jun" is meant the homodimeric complex of the
transcription factor, Jun.

30   Other embodiments of the invention are directed to
methods for screening for potential immunosuppressant
compounds, which interfere with or inhibit lymphokine
gene activation through the NF-AT pathway. One such
method takes advantage of our discovery that the NF-AT$_p$
35   exists in a phosphorylated form in inactivated T cells,

- 6 -

but is dephosphorylated via a phosphatase after
activation through the T cell receptor. This method
involves providing purified, phosphorylated NF-AT$_p$
protein; contacting the NF-AT$_p$ protein with a

5 phosphatase, such as calcineurin or calf intestinal
phosphatase, in the presence of a candidate
immunosuppressive compound; and determining whether
dephosphorylation of NF-AT$_p$ by the phosphatase is
inhibited by the candidate compound.

10   Another method for screening potential
immunosuppressive agents in accordance with the present
invention comprises providing purified NF-AT$_p$;
contacting the purified NF-AT$_p$, in the presence of a
candidate immunosuppressive compound, with an

15 oligonucleotide comprising a sequence substantially
identical to a 5' NF-AT DNA sequence which binds the NF-
AT$_p$ component of the NF-AT complex and determining
whether the candidate compound inhibits binding of the
oligonucleotide. to NF-AT$_p$. In a preferred embodiment,

20 the oligonucleotide is GCCCAAAGAGGAAAATTTGTTTCATACAG (SEQ
ID NO:1).

        Yet another method for screening potential
immunosuppressive agents in accordance with the present
invention involves providing purified NF-AT$_p$; contacting

25 the NF-AT$_p$ with a Fos protein in the presence of a
candidate immunosuppressive compound; and determining
whether the candidate compound inhibits binding of the
Fos protein to the NF-AT$_p$ protein. The same method can
alternatively be used with a Jun protein or a combination

30 of Fos and Jun proteins, and involves providing purified
NF-AT$_p$;
contacting the NF-AT$_p$ with Jun or a combination of Fos
and Jun in the presence of a candidate immunosuppressive
compound; and determining whether the candidate compound

35 inhibits binding of the proteins to the NF-AT$_p$ protein.

- 7 -

Other aspects of the invention will be appreciated by persons skilled in the art from the specification and claims herein.

Brief Description of the Drawing

5   Fig. 1 is a series of photographs of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels showing the identification of T cell-specific NF-AT-binding proteins by renaturation of denatured protein separated and purified from SDS-PAGE

10 gels. The top portion of the figure is a silver stain of an analytical 10% SDS-PAGE gel after fractionation of hypotonic extracts (4 μg total protein) from the murine T cell clone Ar-5 or a murine fibroblast cell line (L cells). The migration of standards and their molecular

15 weights in kDa are indicated above. The bottom portions of the figure illustrate DNA-binding activity of proteins eluted and renatured from a preparative SDS-polyacrylamide gel, and assayed by electrophoretic mobility shift assay (EMSA) using an NF-AT

20 oligonucleotide corresponding to the distal NF-AT site from the murine IL-2 promoter. Lanes 1-18 represent proteins eluted from successive 0.5 cm slices of a preparative SDS gel which was loaded with 200 μg total protein from the same extracts shown on the silver

25 stained analytical gel. The arrow indicates the mobility of a protein-DNA complex detected in T cell extracts but not in L cell extracts. Lanes 6 and 7 contain proteins of apparent molecular weight 107-120 kDa and 93-107 kDa, respectively.

30   Figs. 2A and 2B are photographs of SDS-PAGE gels in which NF-AT-binding proteins were detected in T cell nuclear extracts after renaturation from SDS-polyacrylamide gels. In Fig. 2A, Ar-5 cells were untreated (M), or treated for 2 hours with cross-linked

- 8 -

anti-CD3ε antibody (αCD3), 1 μM CsA, or both anti-CD3ε
and CsA as indicated. Nuclear extracts were prepared and
200 μg of total protein was fractionated by SDS-PAGE,
followed by elution and renaturation of proteins as in
5   Fig. 1. The binding of proteins from three consecutive
gel slices (representing the molecular weight ranges 122-
144, 102-122, and 86-102 kDa as indicated) to the NF-AT
oligonucleotide is shown. In Fig. 1B, specificity of
binding of the renatured proteins to the NF-AT
10 oligonucleotide. Renatured NF-AT-binding proteins from
hypotonic extracts (left panel) or nuclear extracts
(right panel) were assayed for binding to the NF-AT
oligonucleotide in the presence of a 200-fold excess of
unlabeled NF-AT oligonucleotide (here labeled NF-AT-1,
15 lane 2), or oligonucleotides bearing mutations in the NF-
AT sequence (M1, M2, M3, lanes 3-5).

Fig. 3 is a photograph of an EMSA gel showing that
NF-AT-binding proteins derived from V8 protease treatment
of hypotonic or nuclear extracts are similar to those
20 derived from NF-AT$_p$ renatured from SDS-acrylamide gels.
Hypotonic extract, nuclear extract, or NF-AT-binding
protein renatured from hypotonic extract, were mixed with
increasing amounts of V8 protease as indicated, followed
by addition of binding buffer, dI:dC, and labelled NF-AT
25 oligonucleotide for the EMSA. The mixtures were
incubated for 15 minutes at room temperature, and then
subjected to gel electrophoresis at 4°C. Because the gel
was run at 4°C, a more slowly migrating nuclear form of
NF-AT (composed of NF-AT$_p$ plus Fos and Jun proteins) is
30 faint in this gel (lane 5), but the same results are
obtained when electrophoresis is done room temperature
where the larger complex is more apparent.

Figs. 4A and 4B are photographs of an EMSA gel
showing the effect of treatment of NF-AT$_p$ with exogenous
35 phosphatases. A shift in NF-AT$_p$ mobility was observed on

- 9 -

polyacrylamide gels. NP-40 extracts of Ar-5 cells were
ammonium sulfate precipitated to enrich NF-AT$_p$ proteins,
and the resulting proteins were treated with calf
intestinal phosphatase (CIP) (Fig. 4A) or calcineurin
5 (CaN) (Fig. 4B) as detailed below. The proteins were
then fractionated on SDS-PAGE gels, eluted from
individual gel slices spanning the molecular weight
ranges indicated, renatured and assayed for binding to
the NF-AT oligonucleotide. The solid arrow denotes NF-
10 AT$_p$, while the open arrow denotes a minor complex
appearing in the slice containing proteins of molecular
weight 127-143 kDa.

        Figs. 5A through 5C are photographs of EMSA gels
showing that NF-AT$_p$ is a target for CsA. Hypotonic
15 extracts (250 μg total protein) from untreated Ar-5 cells
(Fig. 5A) or cells treated for 10 minutes with 1 μM CsA
(Fig. 5B) were fractionated on SDS-acrylamide gels.
Proteins were eluted and renatured from individual gel
slices, and assayed for binding to the NF-AT
20 oligonucleotide. In Fig. 5C, equal amounts of extracts
(125 μg protein each) from untreated or CsA-treated cells
were mixed after boiling in SDS-PAGE sample buffer and
fractionated as for Figs. 5A and 5B. In each of Figs. 5A
through 5C, the solid arrow denotes NF-AT$_p$, while the
25 open arrow denotes a minor complex appearing in slice 3
(see text for discussion).

        Fig. 6 is a photograph of a portion of an EMSA gel
showing the results of an EMSA. The effect of a
calcineurin inhibitor peptide on the mobility of NF-AT$_p$
30 is shown. Ar-5 cells were lysed by freeze-thawing in
hypotonic buffer containing 100 μM of either a specific
calcineurin inhibitory peptide (IC$_{50}$, 10 μM), or a control
peptide containing a single amino acid substitution that
does not inhibit calcineurin (IC$_{50}$ >200 μM). The extracts
35 (200 μg protein) were fractionated on SDS-acrylamide

- 10 -

gels, and proteins were renatured from gel slices
spanning the molecular weight ranges indicated
(corresponding to lanes 3, 4 and 5 in Figs. 5A-5C), and
assayed for NF-AT binding. Only the portion of the gel
5 shift gel containing the NF-AT$_p$-DNA complexes is shown.

      Fig. 7 is a photograph of an EMSA gel showing the
results of an EMSA. Purified NF-AT$_p$ was shown to
associate in vitro with recombinant Fos and Jun proteins.
Partially purified NF-AT$_p$ was incubated with labeled
10 oligonucleotide corresponding to the distal NF-AT site of
the murine IL-2 promoter, either alone (lanes 1, 7, 13),
with full length or truncated (T) recombinantly produced
c-Fos and c-Jun proteins (lanes 2-5 and 8-11,
respectively) or with an equivalent concentration of
15 bovine serum albumin. Lane 5 contained 50% more c-Fos
and c-Jun compared to lane 4. Lanes 11-13 contain half
the amount of NF-AT$_p$ compared to lanes 1-10. Lane 14
shows a binding reaction of nuclear extracts from
stimulated AR-5 T cells. Bound complexes were separated
20 from free probe by EMSA. The position of free probe, of
the lower (NF-AT$_p$) complex and upper (NF-AT$_p$-Fos-Jun)
complex are indicated.

      Fig. 8 is a photograph of an EMSA gel showing an
analysis of NF-AT$_p$ by renaturation of NF-AT$_p$ activity
25 following purification of the protein using SDS-poly-
acrylamide gel electrophoresis. In the top panel, puri-
fied NF-AT$_p$ (50 ng) was subjected to electrophoresis on
an analytical 6% SDS-PAGE gel and subsequently silver-
stained (Pierce Gel-code kit). In the bottom panel, a
30 second lane of the same gel was loaded with 50 ng of the
purified protein. After electrophoresis, the gel was
sliced, proteins were eluted from gel slices and
renatured, and the fractionated proteins were evaluated
in an EMSA for their ability to bind to the NF-AT site of
35 the murine IL-2 promoter.

- 11 -

Fig. 9 is a photograph of an EMSA gel showing the
results of an EMSA. Antisera to tryptic peptides of
purified NF-AT$_p$ recognize NF-AT$_p$ in T cell extracts.
Cytosolic extracts from unstimulated Ar-5 T cells (lanes
5 1-5) or nuclear extracts from Ar-5 T cells stimulated
with anti-CD3 (lanes 6-10) were incubated without
antiserum (lanes 1 and 6), with antiserum to peptide 72
(residues 206-227 of SEQ ID NO:5, see Fig. 10) (immune,
lanes 3 and 8) or with serum from the same rabbit taken
10 before immunization (preimmune, lanes 2 and 7), then
analyzed by EMSA for binding to the NF-AT
oligonucleotide. For peptide competition, 1 mg of
peptide 72 (lanes 4 and 9) or peptide 25 (residues 685-
703 of SEQ ID NO:5, see Fig. 10) (lanes 5 and 10) was
15 mixed with the antiserum before it was added to cell
extracts. Filled arrowheads identify the cytosolic NF-
AT$_p$, nuclear NF-AT$_p$, and nuclear NF-AT$_p$/Fos/Jun complexes;
open arrowheads indicate the "supershifted" complexes;
the unmarked complex results from binding of serum
20 proteins.

Fig. 10 is a diagram of the deduced amino acid
sequence of murine NF-AT$_p$ (SEQ ID NO:5) in which the
sequences of tryptic peptides from purified NF-AT$_p$ are
underlined. "X" in the underlining for peptides 23.3 and
25 72 indicate positions at which the identity of the amino
acid could not be determined unambiguously. The sequence
between the arrowheads represents the NF-AT$_p$ sequence
contained within the recombinant protein that was
expressed in bacteria.

30   Fig. 11 is an autoradiograph of a Northern blot
analysis of NF-AT$_p$ mRNA from T cell and fibroblast cell
lines. Cytoplasmic RNA from the murine T cell clone Ar-
5, the transformed T cell line C1.7W2, and the murine
fibroblast L cell line were separated by electrophoresis
35 in formaldehyde gels, transferred to nylon membranes and

- 12 -

hybridized with a labelled fragment of NF-AT$_p$ coding
sequence corresponding to the approximately 800-bp PCR
product. The positions of the major NF-AT$_p$ transcript
(arrow) and of 28S and 18S ribosomal RNAs are indicated.

5 The lower panel shows ethidium bromide staining of the
RNA before transfer to nitrocellulose, indicating that
the RNA was intact and that equivalent amounts of RNA
were loaded in each lane.

Fig. 12 is a photograph of an EMSA gel. Binding

10 of a recombinant fragment of NF-AT$_p$ (NF-AT$_p$XS) to target
DNA and association of the recombinant NF-AT$_p$ fragment
with Fos and Jun proteins was evaluated. The binding of
the recombinant fragment of murine NF-AT$_p$ to the distal
NF-AT site of the murine IL-2 promoter was assessed by

15 EMSA in the presence or absence of a 200-fold excess of
unlabelled competitor oligonucleotides as shown in Lane
1-5. The arrows indicate two DNA-protein complexes
formed with NF-AT$_p$XS. Full-length recombinant c-Fos and
c-Jun proteins were included in the same binding

20 reactions, as shown in Lanes 7-9. The open arrows
indicate Jun-Jun-NF-AT$_p$XS complexes, while the closed
arrows indicate Fos-Jun-NF-AT$_p$XS complexes. Fos and Jun
proteins do not bind to the NF-AT oligonucleotide, as
shown in Lane 10.

25 Fig. 13 is a photograph of an EMSA gel showing the
results of an EMSA. Antisera to recombinant NF-AT$_p$
recognize NF-AT$_p$ in T cell extracts. Cytosolic extracts
from unstimulated Ar-5 T cells or nuclear extracts from
cells stimulated with anti-CD3 were incubated without

30 antiserum (lanes 1 and 5), with an antiserum raised
against the recombinant NF-AT$_p$ fragment (lanes 3 and 7),
or with preimmune serum from the same rabbit (lanes 2 and
6), followed by gel shift analysis of binding to the NF-
AT oligonucleotide.

Fig. 14 is a photograph of RNA transcripts
resolved on denaturing polyacrylamide gels and
quantitated using a phosphorimager. Transcriptional
activation by NF-AT$_p$, c-Fos and c-Jun on different

5 templates was analyzed. The templates indicated above
the lanes were incubated with the proteins listed and
transcribed *in vitro* in nuclear extracts from Namalwa
cells. The template pMILNFAT-CAT (lanes 1-14) contains
three NF-AT sites upstream of the basal IL-2 promoter;

10 the template pMILM3-CAT (lanes 15-17) contains four NF-AT
sites in which critical contact residues have been
altered; and the template pCOL-CAT contains residues -73
to +63 of the human collagenase promoter including an AP-
1 site. The filled arrowheads point to the specific

15 transcripts and the open arrowheads to internal controls.
The level of transcription in the presence of different
combinations of proteins was expressed relative to a
reaction in the absence of recombinant proteins (fold
activation). The average of several independent

20 experiments (number shown at the base of each bar) and
the standard deviation in cases in which more than three
independent experiments were performed are shown (solid
bars: activation; open bars: repression).

Fig. 15 is an autoradiograph of a Southern

25 hybridization assay. A labelled probe made from the
EcoRI fragment (described in text) of the murine NF-AT$_p$
cDNA, representing the 5' end of coding sequence
contained in clone mNF-AT$_p$-Q1B1/A, hybridizes to specific
restriction fragments of human genomic DNA.

30 Hybridization of the same probe to restriction fragments
of murine genomic DNA is shown for comparison.

Fig. 16 is an autoradiograph of a Southern
hybridization assay. A labelled probe made from the
SphI-3' fragment (described in text) of the murine NF-AT$_p$

35 cDNA, representing the 3' end of coding sequence common

- 14 -

to all alternatively spliced forms of murine NF-AT$_p$,
hybridizes to specific restriction fragments of human
genomic DNA. Hybridization of the same probe to
restriction fragments of murine genomic DNA is also
5   shown.

Fig. 17 is a representation of a partial cDNA
sequence of human NF-AT$_p$ (SEQ ID NO:11).

Fig. 18 is a representation of the amino acid
sequence deduced from the partial cDNA sequence of human
10 NF-AT$_p$ (SEQ ID NO:12).

Fig. 19 is a diagram showing the comparison of the
murine and human cDNA sequences in the region of overlap
encoding NF-AT$_p$ (SEQ ID NO:8 and SEQ ID NO:22,
respectively).

15   Fig. 20 is a diagram showing murine isoforms of
NF-AT$_p$. Sequences shown ar (1) nucleotides 2201-2260 of
mNF-AT$_p$Q1B1/A sequence, (2) splicing variant mNF-AT$_p$-R3B1,
and (3) splicing variant mNF-AT$_p$-T2B1. The alternatively
spliced forms are identical in sequence to mNF-AT$_p$Q1B1/A
20 in the region up to and including nucleotide 2208 of
mNF-AT$_p$Q1B1/A, shown in lower case letters. While the
coding sequence of mNF-AT$_p$-Q1B1/A continues 3' to the 60-
nucleotide region shown, both variant forms have in-frame
stop codons, shown in boldface type.

25   Fig. 21 is a DNA sequence (SEQ ID NO:19) of the
NF-AT$_p$ gene.

Fig. 22 is a partial cDNA sequence of murine and
NF-AT$_p$ (SEQ ID NO:21) (GenBank U02079) from the plasmid
mNFATp-Q1B1/A. The plasmid mNFATp-Q1B1/A also contains
30 ~150 nucleotides of 3'-untranslated region after the stop
codon.

Fig. 23 is a partial cDNA sequence from the human
NF-AT$_p$ gene (SEQ ID NO:20).

- 15 -

Detailed Description

Purification of NF-AT$_p$

In one embodiment, the invention features a
purified preparation of NF-AT$_p$ protein, which NF-AT$_p$ may
5 be human, murine, bovine, or any other mammalian NF-AT$_p$,
and which may be prepared, for example, from a natural
source, from an expression system expressing an isolated
DNA encoding NF-AT$_p$, or by synthetic means well known to
persons skilled in the art. For example, the protein can
10 be fractionated on a gel by SDS-PAGE, recovered, and then
renatured, as described in the Examples.

A preferred method for purifying the NF-AT$_p$
involves lysing T cells that are rich sources of NF-AT$_p$
protein, precipitating the protein from cell lysates
15 using a salt, such as ammonium sulfate, and purifying the
NF-AT$_p$ protein on heparin-agarose and then on an affinity
column.

The mouse T cell line C1.7W2 (Valge-Archer et al.,
J. Immunol., 145:4355 (1990) has been found to be a rich
20 source of NF-AT$_p$ protein, which can be used in the
purification methods of the invention. Hypotonic
extracts of the cells are prepared from these cells by
lysing about 2.5×10^7 cells/ml in a buffer containing 10
mM Tris (pH 8.0), 50 mM NaCl, 0.05% NP-40, 1 mM EDTA, 100
25 μg/ml aprotinin, 25 μM leupeptin, 2 mM PMSF, 10 mM
iodoacetamide (IAM). The resulting extracts are
centrifuged at low speed to remove the nuclei, then at
100,000 X g for one hour.

After centrifugation, the protein mixture is salt
30 precipitated with an equal volume of appropriate reagent,
which is preferably ammonium sulfate (final 1.5 M) and
the precipitate resuspended in one-tenth volume relative
to cell lysate of buffer, for example buffer A (20 mM
Hepes, pH 7.4, 100 mM NaCl, 2 mM EDTA, 10% glycerol)

- 16 -

containing 100 μg aprotinin, 25 μg leupeptin, 2 mM PMSF, and 10 mM IAM. The lysate is dialyzed versus the buffer A with 0.5 mM DTT.

 The dialyzed extract is further purified by
5 fractionation on heparin-agarose, in accordance with standard procedures. For example, a salt, such as NaCl, is added to the final concentration of lysate and the batch adsorbed onto heparin-agarose. The column is then loaded, washed with buffer (e.g. buffer A containing 0.2
10 M NaCl) and the proteins eluted with salt gradients of 0.2 to 1 M of reagent. Active fractions are pooled and dialyzed versus buffer. The recoveries at this step are typically 25 to 40%.

 The material eluted from the heparin-agarose
15 column is then subjected to two or three rounds of affinity purification using NF-AT oligonucleotides from the promoter region of a mammalian IL-2 gene. A sepharose column conjugated with about 100-200 nM multimerized murine NF-AT oligonucleotides, e.g. as in
20 SEQ ID NO:1 per ml of resin can be used. In a typical purification, after two rounds of affinity chromatography, the specific activity of the NF-AT$_p$ preparation is about 160,000 U/mg of protein, indicating that about 2% of the protein recovered from the column
25 was NF-AT$_p$.

 Higher capacity columns for affinity purification can also be used, in which higher concentrations of NF-AT oligonucleotides are conjugated to the sepharose column. A high capacity column for affinity purification of
30 sequence-specific DNA binding proteins that can be used in accordance with the invention is described in Larson and Verdine, Nucleic Acids Research, 20:3525 (1992). This column is used in the presence of herring sperm DNA.

 The purified protein can be phosphorylated or
35 unphosphorylated, and can be detected on a gel based upon

- 17 -

the molecular weights determined in accordance with the present invention. Thus, the phosphorylated form of native NF-AT$_p$ exhibits a molecular weight about 110-140 kDa, while the dephosphorylated native protein exhibits a

5 molecular weight of about 100-120 kDa.

The invention also includes purified preparations of a complex of NF-AT$_p$ with a Fos protein, a Jun protein, a combination of Fos plus Jun, or any other such protein with which NF-AT$_p$ is naturally functionally associated *in*

10 *vivo*. It is contemplated that a number of members of the Fos and Jun protein families known to persons skilled in the art will bind NF-AT$_p$ and can therefore be used to form the complexes of the invention. c-Fos and c-Jun proteins are particularly suitable. See, Jain et al.,

15 (1992), *supra*. Other proteins that might be complexed with NF-AT$_p$ include other leucine zipper proteins, such as C/EPBs and CREBs. Like Fos and Jun, these proteins are capable of forming homodimers and heterodimers with one another and therefore might associate with NF-AT$_p$ in

20 the same manner as Fos and Jun proteins.

The multicomponent complexes can be used in vitro transcription assays in order to identify other genes under the control of a promoter which responds to the same or structurally similar complex. An in vitro

25 transcription assay that can be used in accordance with the invention is described, for example, in Kretzschmar et al., Genes and Development, 6:761-772 (1992), the pertinent portions of which are hereby incorporated by reference. The complexes are also useful in identifying

30 candidate immunosuppressants which exhibit the ability to inhibit formation or to cause dissociation of the two or three component complex.

- 18 -

Isolation of DNA clones for NF-AT$_p$ and related proteins

Also within the invention are isolated DNAs which encode NF-AT$_p$. By "isolated DNA" is meant that the DNA molecule encodes NF-AT$_p$, but is free of the genes that,

5   in the naturally-occurring genome of the organism from which the DNA of the invention is derived, immediately flank the gene encoding NF-AT$_p$. The term therefore encompasses, for example, a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease

10 treatment, whether such cDNA or genomic DNA fragment is incorporated into a vector, integrated into the genome of the same or a different species than the organism from which it was originally derived, linked to an additional coding sequence to form a hybrid gene encoding a chimeric

15 polypeptide, or independent of any other DNA sequences.

The DNA may be double-stranded or single-stranded, sense or antisense. The isolated DNA of the invention may be under the transcriptional control of a heterologous promoter (i.e., a promoter other than one

20 naturally associated with the NF-AT$_p$ gene), which promoter, for example, may direct the expression of the DNA of the invention in a particular tissue or at a particular stage of development. A cell which contains such isolated DNA may be cultured under conditions

25 permitting the expression of the DNA, providing a method for conveniently manufacturing recombinant NF-AT$_p$. Prokarytic or eukarotic cells can be used to express the protein encoded by the NF-AT$_p$ gene, including bacteria such as <u>E. coli</u>, yeast, insect cells, and mammalian cells

30 such as CHO and COS cell lines well known to persons skilled in the art.

Fragments of the cDNA encoding NF-AT$_p$, e.g., fragments encoding functional domains, can be used to express protein to determine the 3-dimensional structure

of the $NF-AT_p$ transcriptional regulatory complex. For
example, the use of such fragments in competition assays
may be useful in determining the regions of $NF-AT_p$
contacted by the Fos and Jun proteins.

5   By the term "isoform" is meant the product of
alternative splicing of the nucleic acid transcript
encoding $NF-AT_p$.

Antibodies That Bind To $NF-AT_p$

         Purified $NF-AT_p$, or a peptide fragment thereof,
10 may be used to generate by standard methods a monoclonal
or polyclonal antibody capable of binding to $NF-AT_p$. In
one preferred embodiment, a monoclonal antibody is
generated, using the purified $NF-AT_p$ protein to immunize
an appropriate laboratory animal, such as a mouse. The
15 mouse can be, for example, an RBF/DnJ hyperimmune mouse
(Jackson Laboratories, Bar Harbor, ME). Three days after
the final boost with protein, spleens are removed and
spleen cells fused with NSI nonsecreting myeloma cells
using standard protocols. Hybrid cells are selected by
20 growth in medium containing HAT or hypoxanthine and
azaserine. Hybridoma cells secreting antibodies to NF-
AT_p are identified by the ability of their culture
supernatants to: (1) "supershift" or disrupt the $NF-AT_p$-
DNA complex in a gel shift assay; and (2) stain the
25 approximately 120 kd $NF-AT_p$ protein in a Western blot
procedure. Hybrid cells the supernatants of which are
positive in either assay are subcloned by limiting
dilution, and used to produce ascites in (RBF/Dn x BALB
/c) F1 mice.

30   In another preferred embodiment, polyclonal
antisera are generated using $NF-AT_p$ peptides as
immunogens in rabbits. For generation of antisera
against proteolytic fragments of $NF-AT_p$, rabbits were
immunized with synthetic peptides. For example, to

- 20 -

generate peptide 72-specific antibodies, rabbits were
immunized with a 21-amino-acid synthetic peptide
contained within the amino acid sequence of peptide 72
conjugated to the carrier protein KLH.

5   The monoclonal antibodies generated by this
procedure can be used to verify the identity of
recombinant NF-AT$_p$ cDNA clones obtained by expression or
in vitro transcription/translation of the NF-AT$_p$ cDNA
clones. Polyclonal antisera can also be raised against

10 the expressed recombinant NF-AT$_p$ protein. The antibodies
are useful for determining the intracellular location of
NF-AT$_p$: for example, by immunohistochemical staining of
fixed and permeabilized T cells which are left
unstimulated or are stimulated for 5 minutes to two hours

15 with anti-CD3 antibody.

       Such antibodies, or fragments thereof, which bind
to an epitope of NF-AT$_p$ are also useful in a method for
detecting expression of NF-AT$_p$ in a cell or a tissue,
which method includes the steps of contacting proteins of

20 the cell or tissue (e.g., using whole-cell lysates,
proteins extracted from the cytoplasm or nucleus of the
cell, or in situ on a tissue sample) with the antibody,
and detecting immune complex formation using standard
immunoassay techniques such as ELISA.

25   Likewise, a standard Northern blot assay employing
a NF-AT$_p$ cDNA hybridization probe [e.g. full-length,
single-stranded cDNA or a cDNA fragment at least 20
nucleotides in length (preferably at least 50 and more
preferably at least 100) from a portion of the cDNA which

30 is not homologous to any known cDNA sequence] can be used
to ascertain the relative amount of NF-AT$_p$ mRNA in a cell
or a tissue, in accordance with conventional techniques.

       Either method of determining NF-AT$_p$ expression
could be used to identify cells or tissues in which the

- 21 -

level of NF-AT$_p$ expression was higher or lower than
normal, and thus to diagnose certain disease conditions.
For example, a rare form of inherited immune dysfunction
has been attributed to a lack of functional NF-AT$_p$ in the
5 T cells of the patient, while certain cancers and
lymphomas are believed to involve overexpression of the
NF-AT$_p$ gene.

Screening Methods

        The characteristics of purified NF-AT$_p$ disclosed
10 herein form the basis for several different methods for
screening potential immunosuppressive agents. As an
initial screen for potential immunosuppressants,
candidate compounds can take the form of commercially
available proteins or peptides (which are available in
15 libraries from a variety of sources known and available
to the skilled artisan)., or organic or inorganic
compounds, also available in libraries, that bind to the
NF-AT$_p$ protein in either its phosphorylated or
unphosphorylated form. Candidate compounds that bind to
20 NF-AT$_p$ (as determined, for example, in an EMSA) can then
be used in a more detailed screen, as described
hereinafter.
        For example, a candidate compound can be screened
for its ability to inhibit dephosphorylation of NF-AT$_p$,
25 which in turn should result in down regulation of the
lymphokine gene and hence act as an immunosuppressant.
Potential immunosuppressive agents which act by blocking
the dephosphorylation of NF-AT$_p$ can be identified using
any suitable phosphatase that exhibits the ability to
30 dephosphorylate NF-AT$_p$, as described in the Examples
herein. Preferred phosphatases include the calcium and
calmodulin-dependent phosphatase calcineurin and other T
cell phosphatases known to persons skilled in the art.
The screen involves providing purified, phosphorylated

- 22 -

NF-AT$_p$; contacting the NF-AT$_p$ with a T cell phosphatase,
e.g., calcineurin, in the presence of a candidate
immunosuppressive compound; and determining whether
dephosphorylation of NF-AT$_p$ by the phosphatase is
5   inhibited by the candidate compound. Phosphorylation
status of NF-AT$_p$ can be assessed, for example, as
described in Fruman et al., Proc. Natl. Acad. Sci., USA,
89:3686-3690 (May 1992), the pertinent portions of which
are hereby incorporated by reference. See, especially
10   page 3687 of Fruman et al. Alternatively,
phosphorylation status of NF-AT can be evaluated using
SDS-PAGE, as described in the Examples.
          In another screening assay, purified NF-AT$_p$ is
contacted, in the presence of the candidate
15   immunosuppressive compound, with an oligonucleotide
containing a sequence substantially identical to a
mammalian NF-AT nucleotide sequence, preferably a
sequence including GCCCAAAGAGGAAAATTTGTTTCATACAG (SEQ ID
NO:1); and inhibition of binding of the oligonucleotide
20   to NF-AT$_p$ by the candidate compound is ascertained, with
an inhibition of binding being an indication that the
candidate compound is a potential immunosuppressive
agent.
          Other screening assays in accordance with the
25   invention are designed to detect inhibition of binding of
a Fos and/or Jun protein to NF-AT$_p$ by a candidate
immunosuppressive compound. Such assays can be conducted
in the presence or absence of NF-AT DNA. For example, in
a DNA-based assay, incubation of purified or partially
30   purified NF-AT$_p$ with labeled murine NF-AT site (SEQ ID
NO:1) yields a single DNA-protein complex (See Fig. 7,
lanes 1, 7, and 13) corresponding in migration to a
"lower" NF-AT$_p$ complex (Fig. 7, lane 14). Inclusion of
c-Fos and c-Jun proteins in the binding reaction mixture
35   results in the appearance of a second complex (Fig. 7,

lanes 4 and 5) whose migration is identical to that of
the "upper" NF-AT$_p$/Fos/Jun nuclear complex (Fig. 7, lane
14). The complex is not observed when NF-AT$_p$ is
incubated with c-Fos (See Fig. 7, lanes 2,3). In

5   accordance with a DNA-based screening method, candidate
compounds are incubated with a binding mixture including
purified or partially purified NF-AT$_p$, Fos, and Jun and
complex formation is detected in accordance with the
foregoing. Candidate compounds which inhibit the

10 formation of a NF-AT$_p$/Fos/Jun complex can be detected by
viewing migration pattern on the gel.

        Assays for screening candidate immunosuppressants
that inhibit the association of NF-AT$_p$ with Fos and/or
Jun proteins can also be non-DNA based. For example,

15 detection of complex formation or inhibition of Fos
and/or Jun protein binding can be accomplished by
analyzing the mass of the complex formed in the presence
or absence of a candidate compound using a sucrose
gradient, in accordance with established techniques.

20 See, e.g. Current Protocols In Molecular Biology, F.M.
Ausubel. Ed., § 5.3 (Greene Publishing Associates and
Wiley Interscience, N.Y., 1989). Inhibition of binding
of Fos and Jun proteins to NF-AT$_p$ can also be
demonstrated by immunoprecipitating the binding reaction

25 mixture, including a candidate compound, with antibodies
to NF-AT$_p$, Fos, or Jun and detecting the presence or
absence of complex formation. Antibodies to NF-AT$_p$ can
be prepared as described; antibodies to Fos and Jun
proteins are available commercially, and can also be

30 readily prepared by persons skilled in art, using
conventional techniques. Yet another method of
identifying potential immunosuppressants from candidate
compounds that inhibit or interfere with NF-AT complex
formation involves protein cross-linking procedures known

35 in the art. See, e.g. Diamond et al., Science, 249:1266.

- 24 -

In another embodiment, phosphorylation of purified NF-AT$_p$ by its naturally-occurring kinase may form the basis for an assay, with a compound that inhibits such phosphorylation having potentially significant effects on
5 the activity of a T cell.

Other features and advantages of the invention will be apparent from the following Examples.

In the Examples and specification, the following abbreviations are used and have the meanings
10 hereinafter identified: BSA, bovine serum albumin; CIP, calf intestinal phosphatase; CsA, cyclosporin A; DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IL-2, interleukin-2; kDa,
15 kilodalton; NF-AT, nuclear factor of activated T cells; NF-AT$_p$, preexisting subunit of the nuclear factor of activated T cells; NP-40, Nonidet P-40, PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide
20 gel electrophoresis.

METHODS
Cells: The antigen-specific, murine T cell clone Ar-5 (Rao, A., Faas, S.J., and Cantor, H. (1984) *J. Exp. Med.* **159**, 479-494) was maintained in Dulbecco's modified
25 Eagle's medium supplemented with 10% fetal calf serum, 10 mM Hepes, pH 7.4, 2 mM glutamine, 50 μM 2-mercaptoethanol, 50 units/ml penicillin, 50 μg/ml streptomycin, and 5-10 units/ml partially purified rat IL-2 (Collaborative Research). The murine fibroblast L
30 cell line was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10 mM Hepes, pH · 7.4, 50 units/ml penicillin, and 50 μg/ml streptomycin. **Preparation of cell extracts:** To prepare hypotonic extracts, cells were harvested by centrifugation, washed

- 25 -

with phosphate buffered saline and resuspended to 10^8
cells/ml in buffer containing 7.5 mM Tris (pH 7.6), 2 mM
PMSF, 1 mM MgCl2, 0.5 mM DTT, 0.25 mM leupeptin, 0.1 mM
EDTA and 1 mg/ml aprotinin. The cells were lysed by
5 quick-freezing in dry ice and rapid thawing in a room-
temperature water bath, followed by centrifugation at
12,000 x g for 10 minutes. Where indicated, cells were
treated before harvesting with 1 μM CsA (Sandoz Corp.) or
100 nM FK506 (Fujisawa Pharmaceutical) for 10 minutes at
10 37°C.

In an alternative lysis protocol, extracts
enriched for NF-AT$_p$ were prepared by lysing unstimulated
T cells (2.5 x 10^7/ml) in a buffer containing 20 mM Tris
pH 7.5, 10 mM iodoacetamide, 2 mM PMSF, 0.1 mM EDTA, 25
15 μM leupeptin, 100 μg/ml aprotinin and 0.05 % NP-40. The
cell lysates were first centrifuged at 200 x g to remove
nuclei, and then centrifuged further at 100,000 x g for
60'. The 100,000 x g supernatant was made up to 1.5 M in
ammonium sulfate, and the precipitated proteins were
20 collected by centrifugation at 10,000 x g. The protein
pellets were resuspended in buffer containing 100 mM
NaCl, 20 mM Hepes, pH 7.4, 10 mM iodoacetamide, 2 mM
EDTA, 2 mM PMSF, 25 μM leupeptin, 100 μg/ml aprotinin and
10% glycerol, and extensively dialyzed against the same
25 buffer without iodoacetamide and with 0.5 mM DTT.

For preparation of nuclear extracts from activated
T cells, cells were incubated for two hours with cross-
linked anti-CD3ϵ monoclonal antibody (145-2C11, Kubo,
R.T., Born, W., Kappler, J.W., Marrack, P., and Pigeon,
30 M. (1989) *J. Immunol.* **142**, 2736-2742) in the presence or
absence of 1 μM CsA. Activation conditions and
preparation of nuclear extracts was exactly as previously
described (McCaffrey, P.G., Jain, J., Jamieson, C., Sen,
R., and Rao, A. (1992) *J. Biol. Chem.* **267**, 1864-1871).
35 Protein determinations were done by the method of

- 26 -

Bradford (Bradford, M. (1976) *Anal. Biochem.* **72**, 248-254), using bovine serum albumin as a standard.

Treatment of cell extracts with phosphatase in vitro: Ammonium sulfate-precipitated protein (250 μg in 50 μl of

5 buffer described above) were mixed with 1 μl calf intestinal phosphatase (Boehringer-Mannheim, 24 units/μl). For treatment with calcineurin, 250 μg of ammonium sulfate-precipitated protein was made up to 50 μl in 1.5 mM MnCl₂, 0.5 mM EDTA and 15 mM 2-

10 mercaptoethanol. Purified bovine brain calcineurin (King, M.M., and Heiny, L.P. (1987) *J. Biol. Chem.* **262**, 10658-10662) was added to 1 μM; calmodulin (Sigma) was added to 3 μM. All reactions were incubated at 30°C for 10-15 minutes. The reactions were stopped by the

15 addition of SDS-PAGE sample buffer and boiling, and the proteins were fractionated by SDS-PAGE as described below.

Fractionation of cell proteins by SDS-PAGE, elution and renaturation: Hypotonic or nuclear extracts were boiled

20 in SDS-PAGE sample buffer (65 mM Tris, pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, 0.01 mg/ml bromphenol blue) and fractionated on 3 mm thick SDS-polyacrylamide gels by standard methods (Laemmli, U.K. (1970) *Nature* **227**, 680-685). Molecular weight standards (BioRad

25 Laboratories) were also run on the same gel. After electrophoresis, the standards were cut off and separately stained. For recovery of proteins from the gels, slices (1 cm x 0.5 cm) from wet, unfixed gels were crushed and eluted overnight in 50 mM Tris, pH 7.9, 1 mM

30 DTT, 0.2 mM EDTA, 0.1 mM PMSF, 0.1 mg/ml BSA, 2.5% glycerol and 0.1 % SDS (Baeuerle, P., and Baltimore, D. (1988) *Cell* **53**, 211-217). The eluted proteins (250 μl) were precipitated with 4 volumes of acetone at -20°C, washed with methanol at -20°C, air dried and resuspendend

35 in 2.5 μl of a saturated solution of urea. The urea was

- 27 -

diluted with 125 μl of 20 mM Tris, pH 7.6, 10 mM KCl, 2
mM DTT and 0.1 mM PMSF, and the proteins were left
overnight at 4°C to renature. To assess the recovery of
proteins from individual gel slices, aliquots of

5   renatured proteins were electrophoresed on SDS-
polyacrylamide minigels to separate the proteins derived
from cell extracts from the carrier protein (BSA) added
during elution. The gels were then stained using a
sensitive silver stain procedure (Pierce Gelcode color

10 silver stain kit), and the equivalent recovery of
proteins from different gel slices was confirmed.
Electrophoretic mobility shift assay: EMSAs were
performed using 18 μl of renatured proteins in a 30 μl
mix containing 4 mM Hepes, pH 7.4, 84 mM NaCl, 20 mM KCl,

15 0.08 mM EDTA, 9% glycerol, 0.7 mg/ml BSA, 17 μg/ml
poly(dI:dC) and 0.125 ng of ^{32}P-labelled oligonucleotide.
For competition assays, a 200-fold excess of unlabeled
oligonucleotide was added to the reaction. The reactions
were incubated for 15 minutes at room temperature

20 followed by electrophoresis at 4°C on 4%
Tris/borate/EDTA/acrylamide gels. The NF-AT
oligonucleotide used had the sequence
GCCCAAAGAGGAAAATTTGTTTCATACAG (SEQ ID NO:1),
corresponding to the distal NF-AT site from the murine

25 IL-2 promoter (nucleotides -295 to -267 relative to the
transcription start site, (Serfling, E., Barthelmas, R.,
Pfeuffer, I., Schenk, B., Zarius, S., Swoboda, R.,
Mercurio, F., and Karin, M. (1989) *EMBO J.* **8**, 465-473)).
Oligonucleotides bearing mutations in the NF-AT site were

30 as follows: M1, GCCCAAAGAGGAAAATTTGTTT<u>ATAT</u>CAG (SEQ ID
NO:2); M2, GCCCAAAGAGGAAAAT<u>GGA</u>CTTCATACAG (SEQ ID NO:3);
M3, GCCCAAAGA<u>CCTT</u>AATTTGTTTCATACAG (SEQ ID NO:4); where
underlined nucleotides indicate changes made from the NF-
AT sequence.

- 28 -

Analysis of renatured purified NF-AT$_p$:   Cl.7W2 cell
extracts were prepared by NP-40 lysis and ammonium
sulphate precipitation as described above.   The precipi-
tated protein (1.2 g from 10^{11} cells) was dialyzed against
5 buffer A [150 mM NaCl, 20 mM HEPES (pH 7.4), 2 mM EDTA,
0.5 mM DTT, 10% glycerol], supplemented with protease
inhibitors (100 mg/ml aprotinin, 2.5 mM leupeptin and 2
mM PMSF) and loaded onto a 30 ml heparin-agarose column
(Sigma).   The column was washed with 10 column volumes of
10 the same buffer containing 200 mM NaCl, and bound protein
was eluted with a linear gradient of 0.2-1.0 M NaCl in a
total volume of 250 ml.   NF-AT$_p$ activity was determined
by EMSA.   Active fractions were combined and dialyzed
overnight against Buffer A.   The dialyzed pool (90 ml, 95
15 mg protein) was loaded in 20 mg batches onto a 1 ml high
capacity oligonucleotide affinity column in the presence
of 200 μg/ml sheared herring sperm DNA.   The column was
washed with the same buffer, and NF-AT was eluted with a
linear gradient of 0.15-1.0 M NaCl.   The NF-AT activity
20 eluted in fractions between 0.4 M and 0.6 M NaCl. The
peak fractions from several separate fractionations were
combined, dialyzed against buffer containing 150 mM NaCl,
and reloaded onto the same affinity column.   After two
cycles over the affinity column, approximately 10 μg of
25 highly purified NF-AT$_p$ was obtained.   This material bound
specifically to the NF-AT site was a phosphoprotein
substrate for calcineurin, and associated with c-Fos and
c-Jun to form the NF-AT-Fos-Jun ternary complex on the
NF-AT site oligonucleotide.   Renaturation from gel slices
30 and EMSAs were performed as described above.   The
purified NF-AT$_p$ protein was acetone precipitated,
subjected to electrophoresis on a 6% SDS polyacrylamide
gel, and transferred to nitrocellulose.   The NF-AT$_p$ band
was localized by Ponceau Red staining, excised, and
35 digested with trypsin *in situ*.   The resultant peptides

were separated by microbore HPLC. Isolated proteolytic
fragments were then analyzed by laser desorption mass
spectroscopy, and microsequenced using an automated amino
acid analyzer (Edman degradation).

5 **Cloning of the gene encoding murine NF-AT$_p$:** Degenerate
oligonucleotides based on the sequences of peptides 23.2
and 25 were used in a polymerase chain reaction to
amplify an approximately 800-bp fragment from C1.7W2
cDNA. The fragment was used to screen an amplified cDNA
10 library (representing 10^6 primary plaques) in the vector
λZAPII (Stratagene) generated by oligo(dT) and random
priming of cytoplasmic poly(A)$^+$ mRNA from Ar-5 T cells.
After plaque purification of the recombinant λ
bacteriophage clones and excision of pBluescript
15 phagemids carrying the cDNA inserts, the coding sequences
of several cDNA clones were determined by sequencing both
strands using the dideoxy chain termination method well
known in the art.

A cDNA fragment common to all the alternatively
20 spliced cDNAs was excised by digestion with Xho I and Sma
I, subcloned into the vector pQE-31 (Qiagen), and expres-
sed as a hexahistidine-tagged protein in bacteria. The
expressed protein contained an additional 18 vector-
encoded amino acids, MRGSHHHHHHTAPHASSV (SEQ ID NO:6) at
25 the NH$_2$-terminus, and 9 amino acids, VDLEPSLIS (SEQ ID
NO:7) at the COOH-terminus, of the sequence indicated
between the arrowheads in Fig. 10. The recombinant
protein was purified by chromatography on a nickel-
chelate column in 8 M urea, eluting with 250 mM
30 imidazole. After dialysis against buffer A, the protein
was assayed for DNA binding and Fos and Jun association.

In vitro **transcription reactions:** Nuclear extracts were
made from Namalwa cells (ATCC Accession No. CRL 1432) as
35 described above. Fos and Jun proteins and truncated NF-

- 30 -

AT_p (NF-AT_pXS) were used at 500 μM and plasmid templates linearized by EcoRI were used at 80 μg/ml. Transcripts purified from the reactions were analyzed by polyacrylamide gel electrophoresis and quantitated using

5 a phosphorimager.

Characterization of murine NF-AT_p: To characterize the preexisting, DNA-binding component of NF-AT, we have used the murine IL-2 dependent T cell clone Ar-5. Activation of Ar-5 cells with monoclonal antibodies to the T cell

10 antigen receptor or its associated CD3 complex causes the rapid appearance in the nucleus of several inducible transcription factor including NF-AT, which is followed by expression of the IL-2 gene and cell proliferation (Jamieson, C., McCaffrey, P.G., Rao, A., and Sen, R.

15 (1991) *J. Immunol.* **147**, 416-420). Induction of NF-AT subsequent IL-2 gene expression are completely blocked by CsA and FK506 in these cells. We have previously established that unactivated Ar-5 cells contain a protein or proteins, extracted under hypotonic lysis conditions,

20 that can bind in the EMSA to an oligonucleotide comprising the distal NF-AT sequence from the murine IL-2 promoter. Here we will refer to this NF-AT binding activity as NF-AT_p, to designate it as the subunit of NF-AT that is preexisting before T cell activation.

25 **NF-AT_p can be detected as an approximately 120 kDa protein after renaturation from SDS-polyacrylamide gels:** To determine an approximate molecular weight for NF-AT_p, we took advantage of the fact that some DNA-binding proteins retain their ability to bind DNA after elution

30 from SDS-PAGE gels and renaturation (Baeuerele and Baltimore (1988), Cell, 53:211-217 Briggs, M.R., Kadonaga, J.T., Bell, S.P., and Tjian, R. (1986) *Science* **234**, 47-52). Hypotonic extracts from unstimulated Ar-5 T cells were fractionated by SDS-PAGE, followed by elution

35 and renaturation of proteins from individual gel slices

- 31 -

as described. As a negative control, we also tested
extracts from a fibroblast cell line (L cells) that
contained no detectable binding activity specific for the
NF-AT oligonucleotide before fractionation. The
5 renatured proteins were assayed for DNA-binding by EMSA
using an oligonucleotide spanning the distal NF-AT site
of the murine IL-2 promoter. In T cell extracts, binding
activity was detected in several slices containing
proteins of apparent molecular weight 120 kDa and lower
10 (See Fig. 1A, slices 6-16). In L cell extracts, binding
was detected using renatured proteins from the molecular
weight range 66 kDa and lower (slices 11-18 of Fig. 1A).

          Since NF-AT expression has been reported to be
restricted largely to T and B cells (Verweij, C.L.,
15 Guidos, C., and Crabtree, G.R. (1990) *Journal of
Biological Chemistry* **265**, 15788-15795) and we did not
detect NF-AT binding in unfractionated L cell extracts,
we considered the proteins of molecular weight lower than
66 KDa to be unlikely candidates for NF-AT$_p$.

20   Since nuclear extracts from activated T cells were
expected to contain NF-AT$_p$ as part of the multisubunit
nuclear NF-AT complex, we asked whether such extracts
contained NF-AT-binding proteins that were detectable
after renaturation from SDS-polyacryamide gels. Nuclear
25 extracts containing NF-AT were prepared from Ar-5 T cells
stimulated with an activating monoclonal antibody that
reacts with the CD3ϵ subunit of the T cell antigen
receptor/CD3 complex; these extracts were compared with
nuclear extracts from unstimulated cells or cells
30 stimulated in the presence of 1 μM CsA that do not
contain appreciable levels of NF-AT. After fractionation
of nuclear extracts by SDS-PAGE, DNA-binding proteins
were detected primarily in gel slices containing proteins
of apparent molecular weight 102-122 and 86-102 kDa (Fig.
35 2A, lanes 5-12), and weakly in the gel slice containing

- 32 -

proteins of 122-144 kDa (Fig. 2A, lanes 1-4). An
additional faint protein-DNA complex with a slower
mobility was detected in the proteins from the 122-144
kDa range (lane 2); this complex is discussed below.

5   Nuclear extracts from stimulated T cells, which contain
high levels of NF-AT, yielded the highest levels of DNA-
binding proteins after fractionation on SDS-PAGE gels
(compare lane 6 with lanes 5 and 8 of Fig. 2A). The
yield of total protein from gel slices of each sample was

10 equivalent as assessed by SDS-PAGE and silver staining of
the renatured proteins (data not shown; see Methods). In
contrast to the results shown for hypotonic lysates, no
additional DNA-binding proteins were detected in the
lower molecular weight region of fractionated nuclear

15 extracts (data not shown), suggesting that only the
proteins in the 86-144 kDa range of both hypotonic and
nuclear extracts contained NF-AT$_p$.

The renatured DNA-binding proteins (90-140 kDa) from
nuclear and hypotonic extracts were tested for binding

20 specificity for the NF-AT oligonucleotide by competition
with oligonucleotides containing intact or mutated NF-AT
sequences (Fig. 2B). By using these oligonucleotides in
competition assays, we had previously defined a region in
the distal NF-AT sequence critical for binding of both

25 NF-AT and NF-AT$_p$ (Jain et al., Nature, 356:801-804). The
M3 mutation disrupts this region, and thus this
oligonucleotide fails to bind NF-AT (Jain, J., Miner, Z.,
and Rao, A., 1993, J. Immunol. 151:837-848) or to compete
for NF-AT binding to the unmutated oligonucleotide. The

30 M1 mutation has no effect on the ability of the
oligonucleotide to bind NF-AT, while M2 slightly impairs
NF-AT binding. Similarly, the renatured proteins from
either unactivated (hypotonic extract, Fig. 2B) or
activated (nuclear extract, Fig. 2B) Ar-5 cells that

35 bound to the NF-AT oligonucleotide were completely

- 33 -

competed by the NF-AT oligonucleotide itself and by the
M1 oligonucleotide, partially competed by the M2
oligonucleotide, and not competed at all by the M3
oligonucleotide. Thus, the 90-140 kDa proteins renatured
5 from hypotonic extracts of unactivated T cells or nuclear
extracts of activated T cells displayed the same binding
specificity as previously demonstrated for $NF-AT_p$ and NF-
AT. In contrast, the lower molecular weight DNA-binding
proteins from either T cells or L cells were competed
10 poorly by either the intact or mutated NF-AT
oligonucleotides, indicating that they did not interact
specifically with the critical binding region of the NF-
AT sequence defined by M3, and thus did not contain NF-
AT_p.

15   Protease mapping experiments also provided evidence
that the renatured proteins contained $NF-AT_p$. Treatment
of hypotonic or nuclear extracts from Ar-5 cells with
increasing amounts of V8 protease resulted in generation
of two new major DNA-protein complexes in the gel shift
20 assay (Fig. 3, lanes 1-8). These new complexes
presumably represent the binding of proteolytic fragments
of $NF-AT_p$ that retain their ability to interact with DNA.
In agreement with this idea, V8 protease treatment of gel
fractionated, renatured proteins from hypotonic extracts
25 (Fig. 3, lanes 9-12) or nuclear extracts (data not shown)
caused generation of similar DNA-binding fragments. In
addition, treatment of extracts or renatured proteins
with chymotrypsin generated a set of DNA binding
fragments distinct from those generated by V8 but similar
30 to each other. Protease treatment of nuclear extracts
from unactivated cells that do not contain much $NF-AT_p$
(see Fig. 2A) does not result in generation of any new
DNA-protein complexes. In addition, neither V8 nor
chymotrypsin alone had any detectable DNA-binding
35 activity in the gel shift assay. Thus, the new DNA-

- 34 -

protein complexes detected after protease treatment of
crude extracts are most likely derived from NF-AT$_p$, and
the similarity between the pattern seen with nuclear,
hypotonic or renatured proteins is consistent with the
5   idea that the NF-AT-binding proteins in the renatured
samples are related to the NF-AT$_p$ polypeptides detected
in the original nuclear and hypotonic extracts.

    These results indicate that NF-AT$_p$, the DNA-
binding subunit of NF-AT that is present in unstimulated
10 cells and appears in nuclear extracts upon T cell
activation, migrates in SDS gels as a broad band of
apparent molecular weight 90-140 kDa. By measuring the
DNA-binding activity recovered in the 90-140 kDa region
of the SDS-polyacrylamide gel, and comparing it to the
15 activity in the extracts originally loaded, we determined
that the overall yield of NF-AT$_p$ activity in the
renaturation procedure was approximately 3%, a value
consistent with the yield reported previously for elution
and renaturation of the transcription factor Sp1. We
20 could also renature NF-AT$_p$ after non-equilibrium pH
gradient gel electrophoresis (NEPHGE, O'Farrell, P.Z.,
Goodman, H.M., and O'Farrell, P.H. (1977) *Cell* **12**:1133).
In this system, NF-AT$_p$ also migrated as a single broad
band, suggesting that it is composed of a family of
25 related proteins, or post-translationally modified
variants of a single protein, or both.

    NF-AT$_p$ is a phosphoprotein: Since phosphorylation is
a common source of protein size heterogeneity on SDS
gels, and has important functional consequences for DNA-
30 binding proteins, we examined the effect of exogenously
added phosphatase on NF-AT$_p$ in cell extracts. In initial
experiments, hypotonic extracts prepared by freeze-
thawing and incubated for 10 min at 30°C in the presence
or absence of calf intestinal phosphatase yielded NF-AT$_p$
35 migrating in the 90-120 kDa molecular weight range. When

- 35 -

extracts were prepared by an alternative procedure
involving NP-40 lysis and enrichment of NF-AT$_p$ by
ammonium sulfate precipitation, NF-AT$_p$ was recovered
predominantly in the 113 to 127 kDa molecular weight
5   fraction, and also to a small extent in the 127-143 kDa
and 101-113 kDa fractions (Fig. 4A, left panel, -CIP).
Treatment of the extracts with calf intestinal
phosphatase (Fig. 4A, right panel, +CIP) resulted in the
disappearance of the NF-AT$_p$ complex (indicated by solid
10   arrowhead) from the 127-143 kDa molecular weight
fraction, and a concomitant increase in the intensity of
the complex formed with proteins in the 101-113 kDa slice
(Fig. 4A, right panel). An additional faint protein-DNA
complex (Fig. 4A, left panel, open arrowhead) was
15   detected using proteins in the 127-143 kDa molecular
weight fraction. This complex binds the NF-AT
oligonucleotide specifically, as judged by competition
with intact or mutated oligonucleotides, but it does not
shift in mobility on treatment with phosphatases (Fig. 4A
20   and 4B) or CsA (see below). Although its relation to NF-
AT$_p$ is unknown, it serves as a useful control for
evaluating changes in the apparent molecular weight of
NF-AT$_p$. These results demonstrate that NF-AT$_p$ is a
phosphoprotein whose mobility in SDS-polyacrylamide gels
25   can be altered by changes in its phosphorylation state.
     Since NF-AT$_p$ has been proposed to be a target for
the calcium and calmodulin-dependent phosphatase
calcineurin (protein phosphatase 2B), we tested the
effect of this phosphatase on the mobility of NF-AT$_p$ in
30   SDS gels. Treatment of the ammonium sulfate precipitated
proteins with purified bovine brain calcineurin (Fig. 4B)
caused a decrease in the amount of the highest molecular
weight form of NF-AT$_p$ and an increase in the lower
molecular weight form, although the change was not as
35   dramatic as that seen with calf intestinal phosphatase.

- 36 -

These results suggest that NF-AT$_p$ can be a direct
substrate for the phosphatase calcineurin *in vitro.*
**NF-AT$_p$ is a target for immunosuppressive drugs and
calcineurin:** The ability of the immunosuppressive agents
5   CsA and FK-506 to inhibit induction of NF-AT in activated
T cells appears to result from their ability to inhibit
the modification and perhaps the nuclear translocation of
NF-AT$_p$. Recent studies have shown that CsA and FK506, in
complex with their respective binding proteins
10 (immunophilins), can inhibit the activity of calcineurin
in vitro, and also that calcineurin is inhibited in cell
lysates from cells treated with CsA or FK506 prior to
lysis. To test whether the phosphorylation state of NF-
AT$_p$ could be affected by treatment of cells with CsA, we
15 treated cells for 10 minutes with or without CsA prior to
lysis by freeze-thawing, and fractionated the extracts on
SDS-polyacrylamide gels. For these experiments, we chose
to lyse by freeze-thawing because our previous
experiments showed that NF-AT$_p$ in such lysates would be
20 predominantly in the lower molecular weight form (see
Fig. 1 for example), and previous reports demonstrated
that lysates made under similar conditions from human
Jurkat T cells contained active calcineurin. As
expected, extracts from untreated cells contained NF-AT$_p$
25 activity predominantly in gel slices corresponding to a
molecular weight range of 99 to 111 and 111 to 125 kDa
(Fig. 5A, lanes 4 and 5). In contrast, extracts from
CsA-treated cells contained more activity in the 125-140
kDa range, and very little in the 99-111 kDa range (Fig.
30 5B, lanes 3 and 5). Similar results were seen after
treatment of cells with FK506. This change in apparent
molecular weight was specific for NF-AT$_p$, since proteins
eluting in the lower molecular weight areas of the gel
maintained their relative mobilities (see lanes 6-13).
35 In addition, the change was specific for the predominant

- 37 -

NF-AT$_p$ complex (solid arrowhead), and did not affect the
faint upper complex (lane 3, open arrowhead), and thus
was consistent with the sensitivity of the complexes to
CIP and calcineurin treatment (Fig. 4A and 4B). The
5 apparent change in molecular weight of NF-AT$_p$ upon CsA
treatment was not due to the presence of inhibitors in
the renatured proteins that masked binding activity,
since mixed lysates contained activity in each slice in
the range 99-145 kDa (Fig. 5C). The proteins detected in
10 each slice bound specifically as shown by competition,
and were shown to be related by protease mapping.

To test directly whether activation of calcineurin
during cell lysis was causing the appearance of the lower
molecular weight form of NF-AT$_p$, we lysed cells in the
15 presence of a specific peptide inhibitor of calcineurin
(Hashimoto, Y., Perrino, B.A., and Soderling, T.R. (1990)
J. Biol. Chem. 265, 1924-1927) or in the presence of a
mutated peptide that lacked calcineurin inhibitory
activity (Perrino and Soderling, unpublished results).
20 Inhibition of calcineurin activity by inclusion of the
specific peptide inhibitor in the cell lysis buffer
resulted in the appearance of the higher molecular weight
form of NF-AT$_p$, while lysis in the presence of the non-
inhibitory (control) peptide resulted in detection of the
25 lower molecular weight form (Fig. 6). Since our lysis
buffer contained no EGTA, it was conceivable that the
activation of calcineurin was resulting from calcium
release during cell lysis. In agreement with this idea,
inclusion of 2 mM EGTA in the cell lysis buffer resulted
30 in recovery of the higher molecular weight form of NF-AT$_p$
(data not shown). Together, these results are consistent
with the hypothesis that NF-AT$_p$ is a substrate for
calcineurin in cell lysates, and that CsA and FK506 act
to inhibit dephosphorylation of NF-AT$_p$ in cell lysates by
35 inhibiting calcineurin.

- 38 -

Analysis of purified NF-AT$_p$: NF-AT$_p$ was purified from the
Cl.7W2 cell line, a derivative of the murine T cell clone
Ar-5, by ammonium sulfate fractionation followed by
successive chromatography on a heparin-agarose column and
5 an NF-AT oligonucleotide affinity column. A silver-
stained SDS gel of the purified protein showed a major
broad band migrating with an apparent molecular weight of
approximately 120 kDa (Fig. 8, top panel). This band
contains a DNA-binding phosphoprotein that is dephos-
10 phorylated by calcineurin to yield four sharp bands
migrating with apparent molecular weights of
approximately 110-115 kDa. NF-AT$_p$ DNA-binding activity
was demonstrable in protein eluted from the SDS gel and
renatured, and more than 90% of the activity recovered
15 from the gel comigrated with the approximately 120 kDa
band (Fig. 8, lane 7). The faster-migrating complexes
formed with proteins of slightly lower molecular weight
(lanes 8-11) most likely derive from partial proteolysis.
The purified protein binds to the NF-AT site with the
20 appropriate specificity, and forms a DNA-protein complex
with recombinant Fos and Jun.
**Antisera to tryptic peptides binds to NF-AT$_p$ in T cell
extracts:** Antisera to tryptic peptides derived from the
120 kDa protein to confirm that the 120 kDa protein was
25 the preexisting subunit of the T cell transcription
factor NF-AT. When one such antiserum (raised against a
21-residue synthetic fragment containing residues 206-227
of peptide 72 shown in Fig. 10) was included in the
binding reaction, it "supershifted" the NF-AT$_p$-DNA
30 complex formed by the cytosolic fraction from
unstimulated T cells (Fig. 9, lane 3), as well as both
NF-AT complexes formed by nuclear extracts from stimu-
lated T cells (lane 8). The effect of the serum was pre-
vented by preincubation with its cognate peptide (lanes 4
35 and 9), but not by preincubation with a different peptide

- 39 -

(lanes 5 and 10). Preimmune serum had no effect on
binding (lanes 2 and 7). Similar effects were seen with
antisera to peptides 23.1 and 25. These data demonstrate
that the purified protein is NF-AT$_p$.

5   **Identification of a murine NF-AT$_p$ cDNA clone.** In order
to isolate a cDNA clone encoding NF-AT$_p$, degenerate
oligonucleotides based on the sequences of two tryptic
peptides of purified NF-AT$_p$ were used in a polymerase
chain reaction (PCR) to amplify an approximately 800-bp

10 fragment from Cl.7W2 cDNA, and the fragment was used to
screen a cDNA library from murine T cells. The clone
containing the longest cDNA insert, mNF-AT$_p$Q1B1/A,
contains an insert of about 4.5 kb in length, with an
open reading frame extending 2,672 bp from the 5' end of

15 the insert and with about 1.8 kb of 3' untranslated
region that does not extend to the poly(A) tail. The
open reading frame encodes a polypeptide of 890 amino
acids (SEQ ID NO:5) (Fig. 10) that contains eight of nine
tryptic peptides identified by sequencing of purified NF-

20 AT$_p$. The cDNA insert lacks a small amount of coding
sequence at the 5' end, because the predicted molecular
weight of the encoded protein (97 kD) is somewhat smaller
than the apparent molecular weight of dephosphorylated
NF-AT$_p$ (110-115 kD), and because one tryptic peptide from

25 purified NF-AT$_p$ is unaccounted for in the encoded
protein. A search of the GenBank DNA and protein
databases with the Blast algorithm (Altschul et al.,
(1990) *J. Mol. Biol.* **215**:403-410) indicated that the cDNA
encodes a novel protein. A 464-amino-acid fragment

30 containing the DNA-binding domain displayed a limited
similarity to the rel homology domain of human and murine
RelA (p65) (18.9% and 17.8% amino acid identity,
respectively, over 428 amino acids). A preliminary
analysis of additional cDNA clones indicates that T cells

35 express at least three forms of NF-AT$_p$ related to each

- 40 -

other by alternative splicing and differing at their
COOH-termini.

Cell-specific expression of NF-AT$_p$ RNA: Consistent with
the previous demonstration that NF-AT$_p$ protein is present
5 in T cells but not in L cells, the T cell lines Cl.7W2
and Ar-5, but not L cells, were found to express NF-AT$_p$
mRNA (Fig. 11). The PCR fragment of approximately 800 bp
hybridized to a transcript of about 8-9 kb expressed in
the Cl.7W2 T cell line used for purification of NF-AT$_p$
10 (lane 1) and in the untransformed T cell clone Ar-5 used
to generate the cDNA library (lane 2), but did not .
hybridize to any transcript expressed in L cells (lane
3). Two other cDNA probes representing different parts
of the coding region of NF-AT$_p$ gave similar results.
15 Systematic analysis of the tissue distribution of NF-AT$_p$
can be accomplished by Western analysis, quantitative
PCR, or Northern blot analysis.

Binding specificity of recombinant murine NF-AT$_p$: To
test directly whether the cDNA encoded a protein with the
20 characteristics of NF-AT$_p$, the ability of a recombinant
fragment of the protein to bind to the NF-AT site of the
murine IL-2 promoter and to associate with Fos and Jun
was evaluated. Using the QIAexpress Kit (QIAGEN, Inc.),
464-amino-acid fragment of the protein (sequence between
25 arrowheads in Fig. 10) (SEQ ID NO:21) was expressed as a
hexahistidine-tagged protein in bacteria using the pQE31
vector. This recombinant protein bound to the NF-AT
binding site oligonucleotide in a gel shift assay (Fig.
12, lane 1). Its binding specificity was identical to
30 that of authentic T cell NF-AT$_p$, as judged by competition
with excess unlabeled NF-AT binding site oligonucleotide
(lane 2) and the mutant NF-AT oligonucleotides M1-M3
(lanes 3-5). The M1 oligonucleotide (lane 3) is mutated
in four bases remote from the NF-AT binding site, and
35 competes as strongly for binding as the authentic NF-AT

oligonucleotide; the M2 oligonucleotide (lane 4) is
mutated in four bases located between the M1 and M3
regions, and competes with intermediate efficiency; and
the M3 oligonucleotide (lane 5) is mutated in the GGAA

5 tetranucleotide sequence essential for binding of NF-AT$_p$,
and does not compete for binding. Methylation
interference analysis also showed that binding of the
recombinant protein to the NF-AT site required the GGAA
core binding region, as previously demonstrated for

10 NF-AT. Like NF-AT$_p$ purified from T cells, the recom-
binant protein associated with homodimers of c-Jun or
with heterodimers of c-Fos and c-Jun, but not with c-Fos
alone, to form a DNA-protein complex that migrated with
slower mobility than the NF-AT$_p$-DNA complex in an EMSA

15 (lanes 7-9). c-Fos and c-Jun do not bind to the NF-AT
oligonucleotide in the absence of NF-AT$_p$ (lane 10). The
complex containing c-Fos and c-Jun resembled the nuclear
complex of NF-AT$_p$, Fos, and Jun in that its formation was
competed by excess unlabeled AP-1 oligonucleotide. These

20 data indicate that a fragment of NF-AT$_p$ of approximately
50 kDa is sufficient to account for the DNA binding
properties of NF-AT$_p$ and for its ability to associate
with Fos and Jun proteins.

Antisera to recombinant NF-AT$_p$ recognize NF-AT$_p$ in T cell

25 **extracts:** Definitive evidence that the cDNA clone
encodes NF-AT$_p$ was provided by the ability of antisera to
the recombinant protein to react specifically with NF-AT$_p$
from cytosolic or nuclear extracts of T cells. When
serum from a rabbit immunized with the recombinant

30 protein was included in the EMSA, a small proportion of
the NF-AT$_p$-DNA complexes were "supershifted" (Fig. 13,
lane 3) and most of the DNA-protein complexes appeared to
be in large aggregates (lanes 3 and 7). The predominance
of large aggregates probably reflects recognition by the

35 serum of multiple antigenic determinants on NF-AT$_p$.

- 42 -

Preimmune serum from the same rabbit did not alter the
mobility of NF-AT$_p$-DNA and NF-AT-DNA complexes (lanes 2
and 6).

Transcriptional activation by NF-AT$_p$, c-Fos, and c-Jun on

5 **different templates.** To examine the role of the cloned
NF-AT$_p$ protein in transcription, the effect of the recom-
binant NF-AT$_p$ fragment on transcription *in vitro* from a
template containing three NF-AT sites upstream of the
murine IL-2 promoter was tested (Fig. 14). The same

10 plasmid has been used to demonstrate transcriptional
activation *in vivo* in response to stimulation with
antigen (Jain et al., (1993), *J. Immunol.* **151**:837). A
combination of the recombinant NF-AT$_p$ fragment with c-Fos
and c-Jun, or with c-Jun only, activated transcription

15 from this construct (Fig. 14, lanes 2 and 3). In
combination with NF-AT$_p$, a Jun deletion derivative (J91-
334) lacking the amino-terminal repressor domain was a
more potent activator than full-length Jun (lanes 6, 7,
and 14), as previously observed for transcriptional

20 activation by Jun at AP-1 sites (Kerppola et al., (1993),
Mol. Cell Biol. **13**:3782). In contrast, neither the trun-
cated NF-AT$_p$ alone nor AP-1 proteins alone had a signifi-
cant effect (lanes 5, 10, and 11). Truncated Fos and Jun
proteins (F139-200 and J241-334) containing the dimeriza-

25 tion and DNA-binding domains, but lacking transcriptional
activation domains, are able to form a complex with NF-
AT$_p$. However, the truncated proteins did not activate
transcription in conjunction with truncated NF-AT$_p$ (lanes
8 and 9), indicating that the truncated NF-AT$_p$ is not

30 transcriptionally active in the absence of Fos and Jun.
No significant transcriptional activation was observed
when a template containing a mutated NF-AT site incapable
of binding NF-ATp was used (lanes 15-17). Moreover, the
truncated NF-AT$_p$ had no effect on transcription activated

35 by Fos and Jun on a template containing an AP-1 site

- 43 -

(lanes 18-24), consistent with the observation that NF-AT$_p$ does not form a complex with Fos and Jun on the AP-1 site.

These data show that truncated NF-AT$_p$ forms a
5   transcriptionally active complex with Fos and Jun at the IL-2 promoter NF-AT site, and are consistent with the interpretation that NF-AT$_p$ primarily determines the DNA-binding specificity of the NF-AT complex *in vivo*, whereas at least a portion of the transcriptional activity is
10 provided by Fos and Jun. Since the current experiments were performed using a truncated NF-AT$_p$, they do not exclude the possibility that full-length NF-AT$_p$ possesses a transcriptional activation domain that can function in the absence of Fos and Jun. However, there is evidence
15 suggesting that Fos and Jun family proteins are required along with NF-AT$_p$ to activate transcription at the IL-2 promoter NF-AT site *in vivo*, since mutations in the NF-AT site that prevent the association of Fos and Jun with NF-AT$_p$ abolish the function of this site in activated T
20 cells.

The cDNA clone reported herein fulfills four essential criteria defining NF-AT$_p$: the mRNA is expressed in T cells but not in fibroblasts, a recombinant fragment of the protein binds specifically to the NF-AT site, the
25 recombinant protein fragment forms a transcriptionally active complex with Fos and Jun on the NF-AT DNA sequence, and antibodies directed against the recombinant protein recognize NF-AT$_p$ in T cell extracts. The recombinant protein defines a functional 464-amino-acid
30 fragment of NF-AT$_p$ that contains the domains required for DNA binding and for formation of a transcriptionally active complex with Fos and Jun. The cloning of this novel DNA-binding protein makes possible detailed studies of its structure, its interactions with other
35 transcription factors and with specific sites in DNA, its

- 44 -

role in the induction of IL-2 and other cytokine genes,
and its regulation by calcineurin during T cell activa-
tion.

Murine NF-AT$_p$ isoforms: Fig. 19 shows the partial cDNA
5 sequence of murine NF-AT$_p$ from the cDNA insert of the
deposited plasmid, mNF-AT$_p$-Q1B1/A (designated by "m")
Additional cDNA clones of murine NF-AT$_p$ have been
identified. Sequence analysis of the cDNA inserts
revealed the existence of alternatively spliced isoforms
10 of NF-AT$_p$. The alternatively spliced forms that have
been isolated are identical in sequence to mNF-AT$_p$-Q1B1/A
in the region up to and including nucleotide 2208 of mNF-
AT$_p$-Q1B1/A (see Fig. 20).

Cloning of the human homolog of murine NF-AT$_p$: Four
15 fragments of the coding sequence of murine NF-AT$_p$ cDNA
have been characterized for use as probes to isolate the
human cDNA. Two are restriction fragments that can be
prepared from plasmid mNF-AT$_p$-Q1B1/A: a fragment that
extends from the EcoRI site in the multiple cloning site
20 of the vector to the EcoRI site at nucleotide 570 of the
insert ("EcoRI fragment"), and a fragment that extends
from the PstI site at nucleotide 646 of the cDNA to the
PstI site at nucleotide 1169 ("PstI fragment"). The
third fragment which is approximately 800 bp ("~800 bp
25 PCR product") is a cDNA fragment amplified from mouse T
cell cDNA by the polymerase chain reaction (PCR), and
which corresponds to the region between nucleotides 1314
and 2089 in plasmid mNF-AT$_p$-Q1B1/A. The fourth fragment
corresponds to the region between nucleotides 1849 and
30 2089 in the plasmid, and is obtained from the ~800 bp PCR
product by digestion with SphI ("SphI-3' fragment").

        The cDNA probes described above are sufficient to
identify human cDNAs representing the entire coding
sequence of NF-AT$_p$, including cDNAs that have a region in
35 common with these probes and encode isoforms of NF-AT$_p$

- 45 -

that are related by alternative splicing. The fragments
specified for use as probes span the region common to all
the splicing variants of NF-AT$_p$ cDNA that have been
identified in the mouse T cell, with the exception of the

5 extreme 5' end of the coding sequence, a region of the
human cDNA that has been isolated already in human clone
hNF-AT$_p$-21B2. Radiolabelled probes made from the EcoRI
fragment, the PstI fragment, and the SphI-3' fragment
form stable hybrids with a single human gene (under the

10 same conditions used for screening cDNA libraries) as
demonstrated directly by Southern hybridization to
restriction enzyme-digested human genomic DNA. Specific
binding of the EcoRI fragment and the SphI-3' fragment in
a Southern hybridization is shown in Fig. 15 and 16,

15 respectively.

The experiment shown Fig. 15 and 16 indicates that
these murine cDNA probes satisfy the two essential
conditions for their use in screening human cDNA
libraries: the probes form DNA hybrids with the human

20 sequences that are sufficiently stable to survive washing
under moderate stringency or high stringency conditions;
and they do not label multiple DNA fragments in human
genomic DNA, indicating that a high background of false
positive signals (due to nonspecific hybridization or

25 hybridization to repeated sequences) is unlikely to occur
in screening the cDNA libraries.

The EcoRI fragment probe from mouse cDNA has been
used to isolate a partial length cDNA encoding human NF-
AT$_p$ (hNF-AT$_p$-21B2) from a human Jurkat T cell cDNA

30 library, further demonstrating that the murine cDNA
probes can efficiently identify human cDNA clones
containing NF-AT$_p$ coding sequences. Since the EcoRI
fragment identifies a single gene in Southern analysis of
human genomic DNA, it was likely a priori that the hNF-

35 AT$_p$-21B2 cDNA represented human NF-AT$_p$. Direct evidence

- 46 -

that this clone encodes human NF-AT$_p$ is that the
predicted sequence of the encoded protein is ~90%
identical at the amino acid level to that of mouse NF-AT$_p$
(encoded by mNF-AT$_p$-Q1B1/A) in the region of overlap,
5   which extends for 243 amino acids. Further, the coding
sequence of hNF-AT$_p$-21B2 cDNA in the region 5' to its
overlap with the known mouse NF-AT$_p$ sequence encodes a
peptide very closely related (identical at 30/32
positions) to a tryptic peptide of purified NF-AT$_p$
10   protein from mouse T cells. Although this peptide was
known from microsequencing of the purified mouse protein,
it is not encoded in the partial length mouse cDNA mNF-
AT$_p$-Q1B1/A, and was therefore expected to be encoded near
the 5' end of the NF-AT$_p$ coding sequence.
15   In a parallel strategy to identify human NF-AT$_p$
cDNA clones, a fragment of the human cDNA that represents
the 3' end of the partial-length cDNA already obtained
can be used to screen the human T cell cDNA libraries. A
suitable KpnI-EcoRI restriction fragment can be prepared
20   from plasmid hNF-AT$_p$-21B2. This cDNA fragment extends
from a KpnI site in the cDNA insert (corresponding to the
KpnI site at nucleotide 369 of the mouse cDNA) to the
EcoRI site in the multiple cloning site of the
pBluescript vector. This KpnI-EcoRI fragment of the
25   human cDNA overlaps the EcoRI fragment of mouse cDNA, and
offers the slight advantage that replica filters of the
cDNA library can be washed at even higher stringency than
is possible with the mouse cDNA probes.
         Using reagents derived from the mouse cDNA clone
30   mNF-AT$_p$-Q1B1/A and the human cDNA clone hNF-AT$_p$-21B2, the
isolation of a full-length human NF-AT$_p$ cDNA is well
within the skill of those skilled in the art of molecular
biology. For example, radiolabelled cDNA probes made
from the cDNA inserts of murine clone, mNF-AT$_p$-Q1B1/A,
35   and human clone, hNF-AT$_p$-21B2, can be used to identify

- 47 -

and isolate cDNAs, present in cDNA libraries from human T
cells that contain regions with sequence homology to
these cDNAs. The following paragraphs describe murine
cDNA probes that have been demonstrated to form
5   sufficiently stable hybrids with human NF-AT$_p$ DNA for
efficient screening of cDNA libraries; a human cDNA probe
for screening cDNA libraries; the availability of cDNA
libraries from human T cells; the specific methods that
will be used to isolate cDNAs encoding human NF-AT$_p$; and
10 the methods for characterization of these cDNAs.
**Preparation and labelling of cDNA probes for isolation of
full-length human NF-AT$_p$ clone:** Restriction fragments
can be prepared from the plasmids mNF-AT$_p$-Q1B1/A and hNF-
AT$_p$-21B2 by digestion with the restriction enzyme(s)
15 (EcoRI; PstI; or KpnI and EcoRI) specified above, and
purified by agarose gel electrophoresis in low-melting-
temperature agarose, followed by excision of the
appropriate ethidium-bromide-stained band, and recovery
of DNA from the agarose using methods well known in the
20 art (Sambrook et al, *supra*). The purified DNA probe can
then be radiolabelled by a random priming method using a
kit supplied by Boehringer Mannheim, according to a
protocol from Boehringer Mannheim.

The ~800 bp PCR product and its SphI-3' fragment
25 can be prepared using routine methodology in the
following sequence of steps: preparation of RNA;
synthesis of cDNA; PCR amplification; and digestion with
restriction enzyme. RNA can be isolated from mouse T
cells, and enriched for mRNA by selection on oligo(dT)-
30 cellulose (Sambrook et al., *supra*).

cDNA can be synthesized using random
hexanucleotide primers by known methods, briefly
described as follows. In a silanized tube, 1 μg poly(A)$^+$
RNA (obtained by selection on oligo(dT)-cellulose) and
35 100 pmol random hexanucleotides (Pharmacia) are mixed in

- 48 -

a total volume of 10 μl RNase-free water. The tube is
heated at 95°C for 3 min., chilled on ice, and
centrifuged to bring all the liquid to the bottom of the
tube. Then 10 μl of a reaction mixture [2x MMLV buffer
5 (GIBCO-BRL); 20 mM DTT; 2 mM of each of the 2'-
deoxynucleoside 5'-triphosphates dATP, dCTP, dGTP, dTTP
(Ultrapure dNTP Set, Pharmacia); and 20 U/μl MMLV reverse
transcriptase (GIBCO-BRL)] is added, the reaction is
incubated at 22°C for 10 min, and further incubated at
10 42°C for 60 min. PCR amplification primed by the
oligonucleotides shown below is performed in a 100 μl
reaction volume [containing 20 mM tris HCl, pH 8.3 at
20°C; 25 mM KCl; 2 mM MgCl$_2$; 100 μg/ml BSA; 40 pmol of
each oligonucleotide primer; 50 μM of each of the 2'-
15 deoxynucleoside 5'-triphosphates dATP, dCTP, dGTP, dTTP;
2 U Taq polymerase (Perkin-Elmer Cetus); and 1 μl cDNA
from the above cDNA synthesis reaction]. After
preparation of the reaction mixture it is overlaid with
mineral oil, and subjected to the following series of
20 steps in a thermal cycler: 3 min at 72°C; 5 min at 95°C;
30 sec at 55°C; then 35 sequential cycles [2 min at 72°C;
30 sec at 95°C; 30 sec at 55°C]; then 5 min at 72°C.
This typical PCR protocol is based on that recommended by
Innis MA and Gelfand DH, (1990), Optimization of PCRs, In
25 *PCR Protocols*, Innis MA, Gelfand DH, Sninsky JJ, and
White, TJ, editors, Academic Press (San Diego).
Digestion with SphI, purification of the SphI-3' fragment
by agarose gel electrophoresis, and radiolabelling of the
DNA fragment can be performed using procedures described
30 above.

The following sequences of degenerate
oligonucleotide primers can be used in preparation of the
~800 bp PCR product:
5'-CGTTCGGATCCAGTGTT(TC)ATGGAGAA(AG)ACTACA-3' (SEQ ID
35 NO:9)

5'-CGACAGGATCCTG(TC)TGIATIACIGTIGG(GA)TA(CTGA)GC-3' (SEQ
ID NO:10). The upper sequence is that of the sense
strand primer, and the lower sequence that of the
antisense strand primer. "A", "C", "G", and "T" are the
5 standard single-letter abbreviations for the nucleotide
bases; "I" represents inosine; letters enclosed in
parentheses represent sequence degeneracy, and indicate
which bases are present at that position in the
oligonucleotide. Bold type represents the portion of the
10 primer that is expected to anneal to NF-AT$_p$ coding
sequence; the remainder of each oligonucleotide
incorporates a restriction site and a "GC clamp".
Although primers exactly matched to the known sequence of
the murine cDNA would be expected to yield the same
15 product, the PCR conditions described have been developed
for these degenerate primers, and routinely give the
desired product.

        Since the present application discloses both
murine and human NF-AT$_p$ sequence and makes available
20 plasmids containing such sequence, obtaining the complete
sequence of NF-AT$_p$ is a simple matter of applying routine
hybridization techniques to one or more appropriate cDNA
libraries (e;g., T cell cDNA libraries). To ensure that a
full-length cDNA or overlapping cDNAs representing the
25 entire coding sequence will be obtained, more than one
cDNA library of 1-5 million independent clones can be
screened. Several cDNA libraries from human T cells are
available from commercial sources, e.g., Clontech.

        In the event that it becomes necessary to prepare
30 additional cDNA libraries from human T cells for the
purpose of obtaining a full-length cDNA clone, cDNA
libraries with greater than 1 million independent clones
can be prepared either by commercial services for a fee
(for example, Stratagene, Inc. offers this service), or
35 by a researcher in the laboratory using standard

- 50 -

techniques and commercially available reagents (supplied,
for example, by Stratagene, Inc. or by Promega) that have
been optimized for the steps of cDNA synthesis, ligation
to λ bacteriophage arms, packaging into λ bacteriophage
5 particles, and infection of host cells. To ensure that
the entire coding region of the NF-AT$_p$ mRNA is
represented, the cDNA can be synthesized both with
oligo(dT) priming and with random priming.
Methods for screening cDNA libraries: The screening of
10 replica filters of cDNA libraries with radiolabelled cDNA
probes is routine in the art of molecular biology.
Plating of the cDNA library; preparation of replica
filters; hybridization with radiolabelled probe and
washing; identification of positive plaques by alignment
15 of an autoradiograph of the filter with the original
plate; and plaque purification of individual clones that
contain cDNA hybridizing with the probe have been
described in detail by Sambrook et al. *supra*.
Methods for isolation and sequence analysis of cDNAs.
20 The isolation and sequencing of a phage or plasmid clone
previously determined to contain the cDNA of interest,
i.e., human NF-ATp, can be performed using routine
methodology. For example, to isolate NF-ATp cDNA clones
from libraries in the λZAPII vector, a pBluescript
25 plasmid vector carrying the cDNA insert can be excised
according to the protocol that is supplied by the
manufacturer, e.g., Stratagene, Inc., with the vector or
with commercially-available cDNA libraries made in the
vector, λZAPII. Individual colonies of bacteria
30 containing the resulting plasmid can be grown on
ampicillin-containing medium, and plasmid DNA can be
prepared by standard techniques (Sambrook et al, *supra*).
If libraries made in other λ phage vectors are used,
bacteriophage λ vector DNA containing the cDNA insert can
35 be isolated from individual λ clones (after plaque

purification) using known methods (Sambrook et al,
supra), and digested using the appropriate restriction
enzymes to obtain the cDNA insert or cDNA insert
fragments. These cDNAs can then be subcloned into a
5   plasmid vector (e.g., pBluescriptII), and the plasmid DNA
purified by standard techniques. The sequence of each
cDNA insert can be determined (on both strands) using the
dideoxy chain termination method well known in the art
(Sanger et al, *supra*; Sambrook et al, *supra*).

10 **Identification and characterization of a human NF-AT$_p$**
    cDNA clone.
            The plasmid hNF-AT$_p$-21B2 which has been deposited
    with the ATCC contains a cDNA insert representing a
    portion of the coding sequence of human NF-AT$_p$. Fig. 17
15 (SEQ ID NO:11) depicts a partial sequence of the cDNA
    insert of clone hNF-AT$_p$-21B2. "_____" denotes
    approximately 1.9 kb of cDNA insert, expected to be
    principally 5′ untranslated region, that has not yet been
    sequenced. "_" indicates a gap introduced in the human
20 sequence to maintain alignment with the known murine
    sequence in the region of overlap. Fig. 18 (SEQ ID
    NO:12) shows the amino acid sequence deduced from the
    cDNA insert of hNF-AT$_p$-21B2.
            Fig. 19 shows an alignment of the murine
25 (SEQ ID NO:8) and human (SEQ ID NO:22) NF-AT$_p$ cDNA
    sequences in the region of overlap. The region of
    overlap corresponds to 732 bp at the 5′ end of the murine
    cDNA insert. In this figure, "_" indicates identity and
    "." indicates a gap introduced in the human sequence to
30 maintain alignment with the murine sequence. This
    provisional human sequence appears to contain an in-frame
    stop codon, TGA, between bp 650-660 as shown in Fig. 19.
    Considering the homology between the human and murine
    ·sequences and the tryptic peptide data, this in-frame

- 52 -

stop codon probably represents a sequencing error.
Careful sequence analysis of the deposited human cDNA
clone, hNF-AT$_p$-21B2, is likely to resolve the
discrepancy.

5 **Deposit**

Under the terms of the Budapest Treaty on the
International Recognition of the Deposit of
Microorganisms for the Purpose of Patent Procedure,
deposit of the following materials has been made with the
10 American Type Culture Collection (ATCC) of Rockville, MD,
USA.

Applicants' assignees, President and Fellows of
Harvard University and Dana-Farber Cancer Institute,
Inc., represent that the ATCC is a depository affording
15 permanence of the deposit and ready accessibility thereto
by the public if a patent is granted. All restrictions
on the availability to the public of the material so
deposited will be irrevocably removed upon the granting
of a patent. The material will be available during the
20 pendency of the patent application to one determined by
the Commissioner to be entitled thereto under 37 CFR 1.14
and 35 U.S.C. §122. The deposited material will be
maintained with all the care necessary to keep it viable
and uncontaminated for a period of at least five years
25 after the most recent request for the furnishing of a
sample of the deposited plasmid, and in any case, for a
period of at least thirty (30) years after the date of
deposit or for the enforceable life of the patent,
whichever period is longer. Applicants' assignee
30 acknowledges its duty to replace the deposit should the
depository be unable to furnish a sample when requested
due to the condition of the deposit.

Plasmid hNF-AT$_p$21B2 containing the human NF-AT$_p$
sequence and the plasmid mNF-AT$_p$-Q1B1/A containing the
35 murine NF-AT$_p$ sequence have been deposited on October 28,

- 53 -

1993 with the American Type Culture Collection
(Rockville, MD) and have received ATCC designations 75598
and 75597, respectively.

Alternative cloning strategies: Since the amino acid
5 sequence of purified NF-AT$_p$ has been determined,
obtaining a full length NF-AT$_p$ clone and any
alternatively spliced isoforms can be accomplished using
any one of a number of techniques known to those skilled
in the art. Such cloning techniques are described in
10 detail in Molecular Cloning: A Laboratory Manual,
Sambrook et al. 1989, Second Edition, Cold Spring Harbor
Laboratory, Cold Spring Harbor, N.Y. Three well known
alternative cloning strategies are described below.

Based on the amino acid sequence of purified NF-
15 AT$_p$, two PCR primers of approximately 15 nucleotides can
be synthesized corresponding to either end of a length of
a NF-AT$_p$ peptide of approximately 40 amino acid residues.
cDNA can be prepared from 100 mg of total RNA obtained
from a cell previously determined to express NF-AT$_p$,
20 e.g., AR-5 cells. The primers are added to the cDNA with
standard PCR components and the mixture is incubated
under standard PCR conditions. Amplified fragments can
be separated by electrophoresis and subcloned into a
sequencing vector, for example, Bluescript (Stratagene,
25 La Jolla, CA), by blunt end ligation. Alternatively,
restriction enzyme sites can be incorporated into the PCR
primers and the PCR product digested with the appropriate
restriction enzyme and ligated to a vector which has been
digested with a restriction enzyme to produce compatible
30 sticky ends. The PCR-derived NF-AT$_p$ insert DNA can be
sequenced using known methods e.g., the dideoxy-chain-
termination method, utilizing e.g., SEQUENASE® DNA
polymerase (United State Biochemical Corp.) (Sanger F. et
al., (1977), *Proc. Natl. Acad. Sci. USA* 74:5463-5467).
35 DNA fragments so isolated can be used as hybridization

- 54 -

probes to screen for overlapping cDNA inserts in a cDNA
library prepared from cells known to express NF-AT$_p$,
e.g., Ar-5 cells. Positive clones can be isolated and
the DNA sequenced and compared with that of the available

5   amino acid sequence. Oligonucleotide primers
corresponding to bordering vector regions as well as NF-
AT$_p$ primers prepared from previously isolated cDNA clones
can be employed to progressively determine the sequence
of the entire gene.

10   Fragments of DNA containing sequences that
correspond to the amino acid sequence of NF-AT$_p$ can be
recloned into an expression vector, using a variety of
methods known in the art. For example, a recombinant NF-
AT$_p$ polypeptide can be expressed as a fusion protein with

15   maltose binding protein produced in *E. coli*. Using the
maltose binding protein fusion and purification system
(New England Biolabs), the cloned NF-AT$_p$ sequence can be
inserted downstream and in frame of the gene encoding
maltose binding protein (malE), and the malE-NF-AT$_p$

20   fusion protein can then be overexpressed. In the absence
of convenient restriction sites in the NF-AT$_p$, PCR can be
used to introduce restriction sites compatible with the
vector at the 5′ and 3′ end of the cDNA fragment to
facilitate insertion of the cDNA fragment into the

25   vector.

            Following expression of the fusion protein, it can
be purified by affinity chromatography. For example, the
fusion protein can be purified by virtue of the ability
of the maltose binding protein portion of the fusion

30   protein to bind to amylose immobilized on a column.
Alternatively, an antibody specific for the NF-AT$_p$
portion of the fusion protein can be immobilized on a
column and the fusion protein purified by virtue of the
NF-AT$_p$ portion of the protein binding to immobilized NF-

35   AT$_p$ specific antibody or NF-AT oligonucleotide.

- 55 -

To facilitate protein purification, the pMalE
plasmid contains a factor Xa cleavage site upstream of
the site into which the cDNA is inserted into the vector.
Thus, the fusion protein purified as described above can
5   then be cleaved with factor Xa to separate the maltose
binding protein from recombinant NF-AT$_p$. The cleavage
products can be subjected to further chromatography to
purify the NF-AT$_p$ from the maltose binding protein.
The recombinant NF-AT$_p$ can be tested for
10 functional activity, such as binding specifically to the
NF-AT$_p$ site, the ability to associate with c-Fos and C-
Jun to form the NF-AT$_p$-Fos-Jun ternary complex on the NF-
AT$_p$ site oligonucleotide, and the ability to act as a
substrate for calcineurin.
15   Using another cloning strategy, synthetic
degenerate oligonucleotides corresponding to stretches of
10-20 amino acid residues of NF-AT$_p$ can be made using an
oligonucleotide synthesizer. These oligonucleotides can
be used as hybridization probes to screen a cDNA library
20 as described above. Positive clones can be sequenced,
the sequence compared to the known amino acid sequence,
and functional activity encoded by these DNAs tested as
described above.
The gene encoding NF-AT$_p$ can also be identified
25 using expression cloning techniques. In this case, the
binding of NF-AT$_p$-specific antisera or monoclonal
antibodies can be used to screen an expression library.
Antibodies can be raised in an animal, for example, a
rabbit, using as immunogens purified fragments of NF-AT$_p$
30 obtained as described above. These antibodies are
labeled with a suitable label and are then used as probes
to screen an expression library, e.g., a bacteriophage λ
expression library generated by cloning cDNA from cells
previously determined to express NF-AT$_p$. Positive clones
35 are identified based on NF-AT$_p$-specific antibody binding.

- 56 -

Clones identified in this manner can be isolated, sequenced and tested as described above.

- 57 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Rao, Anjana
                                    Hogan, Patrick Gerald
                                    McCaffrey, Patricia
                                    Jain, Jugnu

    (ii) TITLE OF INVENTION: NF-ATp, A T LYMPHOCYTE
DNA-BINDING PROTEIN

    (iii) NUMBER OF SEQUENCES: 22

    (iv) CORRESPONDENCE ADDRESS:

        (A) ADDRESSEE: Fish & Richardson
        (B) STREET: 225 Franklin Street
        (C) CITY: Boston
        (D) STATE: Massachusetts
        (E) COUNTRY: U.S.A.
        (F) ZIP: 02110-2804

    (v) COMPUTER READABLE FORM:

        (A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
        (B) COMPUTER: IBM PS/2 Model 50Z or 55SX
        (C) OPERATING SYSTEM: MS-DOS (Version 5.0)
        (D) SOFTWARE: WordPerfect (Version 5.1)

    (vi) CURRENT APPLICATION DATA:

        (A) APPLICATION NUMBER: 08/145,006
        (B) FILING DATE: October 29, 1993
        (C) CLASSIFICATION:

    (vii) PRIOR APPLICATION DATA:

        (A) APPLICATION NUMBER: 08/017,052
        (B) FILING DATE: February 11, 1993

        (A) APPLICATION NUMBER: 08/006,067
        (B) FILING DATE: January 15, 1993

    (viii) ATTORNEY/AGENT INFORMATION:

        (A) NAME: Fraser, Janis K.
        (B) REGISTRATION NUMBER: 34,819
        (C) REFERENCE/DOCKET NUMBER: 04590/007001

    (ix) TELECOMMUNICATION INFORMATION:

        (A) TELEPHONE: (617) 542-5070
        (B) TELEFAX: (617) 542-8906
        (C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 29
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single

- 58 -

```
        (D) TOPOLOGY:                  linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GCCCAAAGAG GAAAATTTGT TTCATACAG             29


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:     2:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH:                   29
        (B) TYPE:                     nucleic acid
        (C) STRANDEDNESS:             single
        (D) TOPOLOGY:                 linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GCCCAAAGAG GAAAATTTGT TTATATCAG             29


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:     3:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH:                   29
        (B) TYPE:                     nucleic acid
        (C) STRANDEDNESS:             single
        (D) TOPOLOGY:                 linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCCCAAAGAG GAAAATGGAC TTCATACAG             29


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:     4:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH:                   29
        (B) TYPE:                     nucleic acid
        (C) STRANDEDNESS:             single
        (D) TOPOLOGY:                 linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCCCAAAGAC CTTAATTTGT TTCATACAG             29


(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER:     5:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH:                   890
        (B) TYPE:                     amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY:                 linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Gly Ser Ser Ala Ser Phe Ile Ser Asp Thr Phe Ser Pro Tyr Thr Ser
 1               5                   10                  15
```

- 59 -

```
Pro Cys Val Ser Pro Asn Asn Ala Gly Pro Asp Asp Leu Cys Pro Gln
        20                  25                  30

Phe Gln Asn Ile Pro Ala His Tyr Ser Pro Arg Thr Ser Pro Ile Met
        35                  40                  45

Ser Pro Arg Thr Ser Leu Ala Glu Asp Ser Cys Leu Gly Arg His Ser
    50                  55                  60

Pro Val Pro Arg Pro Ala Ser Arg Ser Ser Ser Pro Gly Ala Lys Arg
65                  70                  75                  80

Arg His Ser Cys Ala Glu Ala Leu Val Ala Pro Leu Pro Ala Ala Ser
                85                  90                  95

Pro Gln Arg Ser Arg Ser Pro Ser Pro Gln Pro Ser Pro His Val Ala
            100                 105                 110

Pro Gln Asp Asp Ser Ile Pro Ala Gly Tyr Pro Pro Thr Ala Gly Ser
        115                 120                 125

Ala Val Leu Met Asp Ala Leu Asn Thr Leu Ala Thr Asp Ser Pro Cys
    130                 135                 140

Gly Ile Pro Ser Lys Ile Trp Lys Thr Ser Pro Asp Pro Thr Pro Val
145                 150                 155                 160

Ser Thr Ala Pro Ser Lys Ala Gly Leu Ala Arg His Ile Tyr Pro Thr
            165                 170                 175

Val Glu Phe Leu Gly Pro Cys Glu Gln Glu Glu Arg Arg Asn Ser Ala
        180                 185                 190

Pro Glu Ser Ile Leu Leu Val Pro Pro Thr Trp Pro Lys Gln Leu Val
        195                 200                 205

Pro Ala Ile Pro Ile Cys Ser Ile Pro Val Thr Ala Ser Leu Pro Pro
210                 215                 220

Leu Glu Trp Pro Leu Ser Asn Gln Ser Gly Ser Tyr Glu Leu Arg Ile
225                 230                 235                 240

Glu Val Gln Pro Lys Pro His His Arg Ala His Tyr Glu Thr Glu Gly
            245                 250                 255

Ser Arg Gly Ala Val Lys Ala Pro Thr Gly Gly His Pro Val Val Gln
        260                 265                 270

Leu His Gly Tyr Met Glu Asn Lys Pro Leu Gly Leu Gln Ile Phe Ile
        275                 280                 285

Gly Thr Ala Asp Glu Arg Ile Leu Lys Pro His Ala Phe Tyr Gln Val
    290                 295                 300

His Arg Ile Thr Gly Lys Thr Val Thr Thr Thr Ser Tyr Glu Lys Ile
305                 310                 315                 320

Val Gly Asn Thr Lys Val Leu Glu Ile Pro Leu Glu Pro Lys Asn Asn
            325                 330                 335

Met Arg Ala Thr Ile Asp Cys Ala Gly Ile Leu Lys Leu Arg Asn Ala
        340                 345                 350
```

- 60 -

```
Asp Ile Glu Leu Arg Lys Gly Glu Thr Asp Ile Gly Arg Lys Asn Thr
        355             360             365

Arg Val Arg Leu Val Phe Arg Val His Val Pro Glu Pro Ser Gly Arg
    370             375             380

Ile Val Ser Leu Gln Ala Ala Ser Asn Pro Ile Glu Cys Ser Gln Arg
385             390             395             400

Ser Ala His Glu Leu Pro Met Val Glu Arg Gln Asp Met Asp Ser Cys
            405             410             415

Leu Val Tyr Gly Gly Gln Gln Met Ile Leu Thr Gly Gln Asn Phe Thr
            420             425             430

Ala Glu Ser Lys Val Val Phe Met Glu Lys Thr Thr Asp Gly Gln Gln
        435             440             445

Ile Trp Glu Met Glu Ala Thr Val Asp Lys Asp Lys Ser Gln Pro Asn
450             455             460

Met Leu Phe Val Glu Ile Pro Glu Tyr Arg Asn Lys His Ile Arg Val
465             470             475             480

Pro Val Lys Val Asn Phe Tyr Val Ile Asn Gly Lys Arg Lys Arg Ser
            485             490             495

Gln Pro Gln His Phe Thr Tyr His Pro Val Pro Ala Ile Lys Thr Glu
            500             505             510

Pro Ser Asp Glu Tyr Glu Pro Ser Leu Ile Cys Ser Pro Ala His Gly
        515             520             525

Gly Leu Gly Ser Gln Pro Tyr Tyr Pro Gln His Pro Met Leu Ala Glu
        530             535             540

Ser Pro Ser Cys Leu Val Ala Thr Met Ala Pro Cys Gln Gln Phe Arg
545             550             555             560

Ser Gly Leu Ser Ser Pro Asp Ala Arg Tyr Gln Gln Gln Ser Pro Ala
            565             570             575

Ala Ala Leu Tyr Gln Arg Ser Lys Ser Leu Ser Pro Gly Leu Leu Gly
            580             585             590

Tyr Gln Gln Pro Ser Leu Leu Ala Ala Pro Leu Gly Leu Ala Asp Ala
        595             600             605

His Arg Ser Val Leu Val His Ala Gly Ser Gln Gly Gln Gly Gln Gly
    610             615             620

Ser Thr Leu Arg His Thr Ser Ser Ala Ser Gln Ala Ser Pro Val
625             630             635             640

Ile His Tyr Ser Pro Thr Asn Gln Gln Leu Arg Gly Gly Gly His Gln
            645             650             655

Glu Phe Gln His Ile Met Tyr Cys Glu Asn Phe Gly Pro Ser Ser Ala
            660             665             670

Arg Pro Gly Pro Pro Pro Ile Asn Gln Gly Gln Arg Leu Ser Pro Gly
        675             680             685
```

- 61 -

Ala Tyr Pro Thr Val Ile Gln Gln Gln Thr Ala Pro Ser Gln Arg Ala
    690   695   700

Ala Lys Asn Gly Pro Ser Asp Gln Lys Glu Ala Leu Pro Thr Gly Val
705   710   715   720

Thr Val Lys Gln Glu Gln Asn Leu Asp Gln Thr Tyr Leu Asp Asp Ala
            725   730   735

Ala Thr Ser Glu Ser Trp Val Gly Thr Glu Arg Tyr Ile Glu Arg Lys
            740   745   750

Phe Trp Lys Lys Thr Leu Val Gln Pro Gly Leu Leu Pro Ser Phe Leu
        755   760   765

Leu Leu Gly Ser Leu Ser Ala Gly Pro Arg Ser Gln Thr Pro Ser Glu
    770   775   780

Arg Lys Pro Ile Glu Glu Asp Val Pro Leu Ser Cys Ser Gln Ile Ala
785   790   795   800

Trp Cys Cys Gln His Pro Leu Gly Thr Cys Pro Val Leu Pro Gly Pro
            805   810   815

Leu Ala Val Glu Trp Trp Glu Gly Gln Leu Gly Arg Gly Leu Glu Pro
        820   825   830

Ile Pro Trp Ala Pro Asp Ser Ala Gly Ser Leu His Glu Val Asp Ser
        835   840   845

Val Gly Leu Ala Gly Val Val Gly Met Val Leu Leu Thr Leu Met His
    850   855   860

His Phe Ser Met Asp Gln Asn Gln Thr Pro Ser Pro His Trp Gln Arg
865   870   875   880

His Lys Glu Val Ala Ser Pro Gly Trp Ile
            885   890

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

        (i) SEQUENCE CHARACTERISTICS:

            (A) LENGTH: 18
            (B) TYPE: amino acid
            (C) STRANDEDNESS:
            (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Arg Gly Ser His His His His His Thr Ala Pro His Ala Ser
  1   5   10   15

Ser Val

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 7:

        (i) SEQUENCE CHARACTERISTICS:

            (A) LENGTH: 9

- 62 -

```
                (B) TYPE:                          amino acid
                (C) STRANDEDNESS:
                (D) TOPOLOGY:                       linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:.7:

Val Asp Leu Glu Pro Ser Leu Ile Ser
 1               5
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 8:

    (i) SEQUENCE CHARACTERISTICS:

```
                (A) LENGTH:                        732
                (B) TYPE:                          nucleic acid
                (C) STRANDEDNESS:                  double
                (D) TOPOLOGY:                       linear
```

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
GCGGCTCCTC TGCCAGCTTC ATTTCTGACA CCTTCTCCCC CTACACCTCG CCCTGCGTCT    60

CACCCAATAA CGCCGGGCCC GACGACCTGT GTCCCCAGTT TCAAAACATC CCTGCTCATT   120

ATTCCCCCAG AACCTCTCCA ATAATGTCAC CTCGAACCAG CCTCGCCGAG GACAGCTGCC   180

TGGGCCGACA CTCGCCCGTG CCCCGTCCGG CATCCCGCTC CTCCTCACCC GGTGCCAAGC   240

GGAGGCATTC GTGCGCAGAG GCTTTGGTTG CTCCTCTGCC CGCAGCCTCA CCCCAGCGCT   300

CCCGGAGCCC CTCGCCACAG CCCTCGCCTC ACGTGGCACC GCAGGACGAC AGCATCCCCG   360

CTGGGTACCC CCCCACGGCC GGCTCTGCTG TTCTCATGGA TGCCCTCAAC ACCCTGGCCA   420

CCGACTCGCC CTGCGGGATC CCCTCCAAGA TATGGAAGAC CAGTCCTGAC CCGACGCCTG   480

TGTCCACCGC TCCGTCCAAG. GCTGGCCTGG CCCGCCACAT CTACCCTACT GTGGAGTTCC   540

TGGGGCCATG TGAGCAGGAG GAGAGGAGGA ATTCCGCTCC AGAGTCCATC CTGCTGGTAC   600

CACCTACTTG GCCCAAGCAG TTGGTGCCGG CCATTCCCAT CTGCAGCATC CCTGTGACTG   660

CATCCCTCCC ACCACTCGAG TGGCCACTCT CCAATCAGTC GGGCTCCTAT GAGCTACGGA   720

TTGAGGTCCA AC                                                       732
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 9:

    (i) SEQUENCE CHARACTERISTICS:

```
                (A) LENGTH:   .                    33
                (B) TYPE:                          nucleic acid
                (C) STRANDEDNESS:                  single
                (D) TOPOLOGY:                       linear
```

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

```
CGTTCGGATC CAGTGTTYAT GGAGAARACT ACA      33
```

- 63 -

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 10:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 34
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CGACAGGAT CCTGYTGNATN ACNGTNGGRT ANGC   34

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 11:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 432
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCCGGAGCCC CTCGCCGCAG CCCTCATCTC ACGTGGCACC CCAGGACCAC GGCTCCCCGG   60

CTGGGTACCC CCCTGTGGCT GGCTCTGCCG TGATCATGGA TGCCCTGAAC AGCCTCGCCA   120

CGGACTCGCC TTGTGGNATC CCCCCCAAGA TGTGGAAGAC CAGCCCTGAC CCCTCGCCGG   180

TGTCTCGCGC CCCATCCAAG GCNGGCCTGC CTCGCCACAT CTACCCGGCC GTGGAGTTCC   240

TGGGGCNNTG CGAGCAGGGC GAGAGGAGAA ACTCGGCTCC AGAATCCATC CTGCTGGTTC   300

CGCCCACTTG NCCCAAGCCG CTGGTGCCTG CCATTCCCAT CTCGACGATC CCATGAGCTC   360

GATCCCTCCC TNCACTTGAG TGGCCGCTGT CCAGTCAGTC ATCGCGTTAC GAGCTGCGGA   420

TCGAGGTGCA GC   432

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 12:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 357
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Ala Tyr Pro Asp Asp Val Leu Asp Tyr Gly Leu Lys Pro Tyr Ser Pro
1   5   10   15

Leu Ala Ser Leu Ser Gly Glu Pro Pro Gly Arg Phe Gly Glu Pro Asp
        20   25   30

Arg Val Gly Pro Gln Lys Phe Leu Ser Ala Ala Lys Pro Ala Gly Ala
        35   40   45

- 64 -

```
Ser Gly Leu Ser Pro Arg Ile Glu Ile Thr Pro Ser His Glu Leu Ile
    50              55              60
```

```
Gln Ala Val Gly Pro Leu Arg Met Arg Asp Ala Gly Leu Leu Val Glu
65              70              75                  80
```

```
Gln Pro Pro Leu Ala Gly Val Ala Ala Ser Pro Arg Phe Thr Leu Pro
            85              90              95
```

```
Val Pro Gly Phe Glu Gly Tyr Arg Gln Pro Leu Cys Leu Ser Pro Ala
        100             105             110
```

```
Ser Ser Gly Ser Ser Ala Ser Phe Ile Ser Asp Thr Phe Ser Pro Tyr
        115             120             125
```

```
Thr Cys Pro Cys Val Ser Pro Asn Asn Gly Gly Pro Asp Asp Leu Cys
    130             135             140
```

```
Pro Gln Phe Gln Asn Ile Pro Ala His Tyr Ser Pro Arg Thr Ser Pro
145             150             155             160
```

```
Ile Met Ser Pro Arg Thr Ser Leu Ala Glu Asp Ser Cys Leu Gly Arg
                165             170             175
```

```
His Ser Pro Val Pro Arg Pro Ala Ser Arg Ser Ser Ser Pro Gly Ala
            180             185             190
```

```
Lys Arg Arg His Ser Cys Ala Glu Ala Leu Val Ala Leu Pro Pro Gly
        195             200             205
```

```
Ala Ser Pro Gln Arg Ser Arg Ser Pro Ser Pro Gln Pro Ser Ser His
    210             215             220
```

```
Val Ala Pro Gln Asp His Gly Ser Pro Ala Gly Tyr Pro Pro Val Ala
225             230             235             240
```

```
Gly Ser Ala Val Ile Met Asp Ala Leu Asn Ser Leu Ala Thr Asp Ser
                245             250             255
```

```
Pro Cys Gly Ile Pro Pro Lys Met Trp Lys Thr Ser Pro Asp Pro Ser
            260             265             270
```

```
Pro Val Ser Arg Ala Pro Ser Lys Ala Gly Leu Pro Arg His Ile Tyr
        275             280             285
```

```
Pro Ala Val Glu Phe Leu Gly Pro Cys Glu Gln Gly Glu Arg Arg Asn
290             295             300
```

```
Ser Ala Pro Glu Ser Ile Leu Leu Val Pro Pro Thr Trp Pro Lys Pro
305             310             315             320
```

```
Leu Val Pro Ala Ile Pro Ile Ser Thr Ile Pro Xaa Ala Arg Ser Leu
                325             330             335
```

```
Pro Pro Leu Glu Trp Pro Leu Ser Ser Gln Ser Ser Arg Tyr Glu Leu
            340             345             350
```

```
Arg Ile Glu Val Gln
            355
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 13:

- 65 -

(i) SEQUENCE CHARACTERISTICS:

    (A) LENGTH: 60
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: double
    (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GGATGACGCA GCCACTTCAG AAAGCTGGGT TGGGACAGAA AGGTATATAG AGAGAAAATT   60

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 14:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 52
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GGATGACGAG TTGATAGACA CACACCTTAG CTGGATACAA AACATATTAT GA   52

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 15:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 60
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GGATGACGTT AATGAAATCA TCAGGAAGGA GTTTTCAGGA CCTCCCTCCC GAAATCAGAC   60

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 16:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 16
         (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Asp Asp Ala Ala Thr Ser Glu Ser Trp Val Gly Thr Glu Arg Tyr Ile
 1   5   10   15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 17:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 16
         (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

- 66 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Asp Asp Glu Leu Ile Asp Thr His Leu Ser Trp Ile Gln Asn Ile Leu
1   5   10   15

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 18:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 20
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Asp Asp Val Asn Glu Ile Ile Arg Lys Glu Phe Ser Gly Pro Pro Ser
1   5   10   15

Arg Asn Gln Thr
            20

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 19:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 1474
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AGCAGGAAGC TCGCGCCGCC GTCGCCGCCG CCGCTCAGCT TCCCCGGGCG CGTCCAGGAC   60

CCGCTGCGCC AGGCGCGCCG TCCCCGGACC CGGCGTGCGT CCCTACGAGG AAAGGGACCC   120

CGCCGCTCGA GCCGCCTCCG CCAGCCCCAC TGCGAGGGGT CCCAGAGCCA GCCGCGCCCG   180

CCCTCGCCCC CGGCCCCGCA GCCTTCCCGC CCTGCGCGCC ATGAACGCCC CCGAGCGGCA   240

GCCCCAACCC GACGGCGGGG ACGCCCCAGG CCACGAGCCT GGGGGCAGCC CCCAAGACGA   300

GCTTGACTTC TCCATCCTCT TCGACTATGA GTATTTGAAT CCGAACGAAG AAGAGCCGAA   360

TGCACATAAG GTCGCCAGCC CACCCTCCGG ACCCGCATAC CCCGATGATG TCCTGGACTA   420

TGGCCTCAAG CCATACAGCC CCCTTGCTAG TCTCTCTGGC GAGCCCCCCG GCCGATTCGG   480

AGAGCCGGAT AGGGTAGGGC CGCAGAAGTT TCTGAGCGCG GCCAAGCCAG CAGGGGCCTC   540

GGGCCTGAGC CCTCGGATCG AGATCACTCC GTCCCACGAA CTGATCCAGG CAGTGGGGCC   600

CCTCCGCATG AGAGACGCGG GCCTCCTGGT GGAGCAGCCG CCCCTGGCCG GGGTGGCCGC   660

CAGCCCGAGG TTCACCCTGC CCGTGCCCGG CTTCGAGGGC TACCGCGAGC CGCTTTGCTT   720

GAGCCCCGCT AGCAGCGGCT CCTCTGCCAG CTTCATTTCT GACACCTTCT CCCCCTACAC   780

CTCGCCCTGC GTCTCGCCCA ATAACGGCGG GCCCGACGAC CTGTGTCCGC AGTTTCAAAA   840

– 67 –

```
CATCCCTGCT CATTATTCCC CCAGAACCTC GCCAATAATG TCACCTCGAA CCAGCCTCGC   900

CGAGGACAGC TGCCTGGGCC GCCACTCGCC CGTGCCCCGT CCGGCCTCCC GCTCCTCATC   960

GCCTGGTGCC AAGCGGAGGC ATTCGTGCGC CGAGGCCTTG GTTGCCCTGC CGCCCGGAGC  1020

CTCACCCCAG CGCTCCCGGA GCCCCTCGCC GCAGCCCTCA TCTCACGTGG CACCCCAGGA  1080

CCACGGCTCC CCGGCTGGGT ACCCCCCTGT GGCTGGCTCT GCCGTGATCA TGGATGCCCT  1140

GAACAGCCTC GCCACGGACT CGCCTTGTGG GATCCCCCCC AAGATGTGGA AGACCAGCCC  1200

TGACCCCTCG CCGGTGTCTG CCGCCCCATC CAAGGCCGGC CTGCCTCGCC ACATCTACCC  1260

GGCCGTGGAG TTCCTGGGGC CCTGCGAGCA GGGCGAGAGG AGAAACTCGG CTCCAGAATC  1320

CATCCTGCTG GTTCCGCCCA CTTGGCCCAA ACCGCTGGTG CCTGCCATTC CCATCTGCAG  1380

CATCCCAGTG ACTGCATCCC TCCCTCCACT TGAGTGGCCG CTGTCCAGTC AGTCAGGCTC  1440

TTACGAGCTG CGGATCGAGG TGCAGCCTCG TGCC                                1474
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 20:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 2675
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

```
GCGGCTCCTC TGCCAGCTTC ATTTCTGACA CCTTCTCCCC CTACACCTCG CCCTGCGTCT    60

CACCCAATAA CGCCGGGCCC GACGACCTGT GTCCCCAGTT TCAAAACATC CCTGCTCATT   120

ATTCCCCCAG AACCTCTCCA ATAATGTCAC CTCGAACCAG CCTCGCCGAG GACAGCTGCC   180

TGGGCCGACA CTCGCCCGTG CCCCGTCCGG CATCCCGCTC CTCCTCACCC GGTGCCAAGC   240

GGAGGCATTC GTGCGCAGAG GCTTTGGTTG CTCCTCTGCC CGCAGCCTCA CCCCAGCGCT   300

CCCGGAGCCC CTCGCCACAG CCCTCGCCTC ACGTGGCACC GCAGGACGAC AGCATCCCCG   360

CTGGGTACCC CCCCACGGCC GGCTCTGCTG TTCTCATGGA TGCCCTCAAC ACCCTGGCCA   420

CCGACTCGCC CTGCGGGATC CCCTCCAAGA TATGGAAGAC CAGTCCTGAC CCGACGCCTG   480

TGTCCACCGC TCCGTCCAAG GCTGGCCTGG CCCGCCACAT CTACCCTACT GTGGAGTTCC   540

TGGGGCCATG TGAGCAGGAG GAGAGGAGGA ATTCCGCTCC AGAGTCCATC CTGCTGGTAC   600

CACCTACTTG GCCCAAGCAG TTGGTGCCGG CCATTCCCAT CTGCAGCATC CCTGTGACTG   660

CATCCCTCCC ACCACTCGAG TGGCCACTCT CCAATCAGTC GGGCTCCTAT GAGCTACGGA   720

TTGAGGTCCA ACCCAAGCCC CATCACCGGG CCCACTATGA GACGGAGGGC AGCCGTGGCG   780

CTGTCAAAGC CCCAACAGGA GGACACCCTG TGGTGCAGCT CCACGGCTAC ATGGAGAACA   840

AGCCTCTGGG GCTTCAGATC TTCATTGGGA CAGCAGATGA GAGGATCCTT AAGCCGCACG   900
```

- 68 -

```
CCTTCTACCA AGTACACAGG ATCACTGGGA AAACGGTCAC CACCACGAGC TATGAGAAGA  960
TCGTAGGCAA CACCAAGGTC CTGGAGATCC CCCTGGAGCC AAAGAACAAC ATGAGAGCCA 1020
CCATCGACTG TGCAGGCATC CTGAAGCTCC GAAACGCTGA CATCGAGCTG CGGAAGGGCG 1080
AGACGGACAT CGGCAGGAAG AACACGCGTG TGCGCCTGGT GTTCCGCGTG CACGTCCCAG 1140
AGCCCAGTGG GCGCATCGTC TCCCTGCAGG CTGCGTCCAA CCCCATCGAG TGCTCTCAGC 1200
GCTCTGCCCA CGAGCTGCCC ATGGTGGAGA GACAAGACAT GGACAGCTGC CTGGTCTACG 1260
GGGGCCAGCA GATGATCCTC ACGGGCCAGA ACTTCACAGC GGAGTCCAAG GTTGTGTTCA 1320
TGGAGAAGAC TACAGATGGG CAGCAGATTT GGGAGATGGA AGCTACGGTG GATAAAGACA 1380
AGAGCCAGCC TAACATGCTT TTTGTTGAGA TCCCCGAGTA TCGGAACAAG CACATCCGCG 1440
TGCCCGTGAA AGTCAACTTC TACGTCATCA ACGGAAAGAG GAAACGAAGT CAGCCACAGC 1500
ACTTTACCTA CCACCCAGTC CCTGCCATCA AGACAGAGCC CAGCGATGAG TATGAACCAT 1560
CTTTGATCTG CAGCCCCGCC CATGGAGGCC TGGGGAGCCA GCCATATTAC CCACAGCACC 1620
CAATGCTGGC CGAGTCCCCC TCCTGCCTTG TGGCTACCAT GGCCCCCTGC CAACAGTTCC 1680
GCTCGGGGCT CTCATCCCCC GATGCTCGCT ACCAACAGCA GAGCCCCGCA GCTGCCCTCT 1740
ACCAGAGAAG CAAGAGCCTG AGTCCCGGCC TGCTGGGCTA CCAGCAGCCG TCCCTCCTGG 1800
CAGCACCCTT GGGTCTGGCT GATGCCCACC GCTCTGTGCT GGTGCATGCT GGTTCTCAGG 1860
GGCAGGGGCA GGGCTCCACC CTCCGACACA CATCCTCGGC CAGCCAGCAG GCCTCACCCG 1920
TGATCCACTA CTCACCCACC AACCAGCAGC TTCGCGGTGG GGGTCACCAG GAGTTCCAGC 1980
ATATCATGTA CTGTGAAAAC TTCGGCCCCA GCTCTGCCAG GCCTGGCCCG CCTCCCATCA 2040
ACCAAGGTCA GAGGCTGAGC CCGGGCGCCT ACCCCACAGT CATCCAACAA CAGACTGCCC 2100
CGAGCCAAAG AGCTGCCAAA AACGGACCCA GTGACCAGAA GGAAGCTCTG CCCACGGGAG 2160
TGACCGTCAA ACAGGAACAG AACCTGGACC AGACCTACCT GGATGACGCA GCCACTTCAG 2220
AAAGCTGGGT TGGGACAGAA AGGTATATAG AGAGAAAATT TTGGAAGAAG ACCCTTGTGC 2280
AGCCTGGGCT CCTGCCCTCA TTTTTACTTC TTGGCTCCCT GTCTGCTGGA CCAAGGTCAC 2340
AGACACCATC AGAAAGAAAG CCCATAGAGG AAGACGTGCC CTTGAGTTGC AGCCAGATAG 2400
CCTGGTGTTG TCAGCATCCC TTGGGGACCT GCCCTGTCCT GCCAGGGCCT TTAGCTGTAG 2460
AGTGGTGGGA AGGGCAGCTC GGGCGTGGGC TGGAGCCAAT TCCCTGGGCT CCAGACAGTG 2520
CCGGCAGCCT CCATGAGGTG GACAGTGTAG GCCTGGCGGG AGTGGTCGGA ATGGTTCTGC 2580
TCACTCTTAT GCACCACTTC TCCATGGATC AGAACCAGAC CCCCTCTCCT CACTGGCAAA 2640
GGCACAAAGA GGTTGCTAGC CCAGGCTGGA TCTGA                            2675
```

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 21:

- 69 -

(i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 113
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: double
      (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

ATGGGCTCAT CAACTTCATC AAGCAGCAGC GCGAGGCCAG AGTCCAATAA ACTCGTGCTC   60

ATCTGCAGCC TCCTCTGTGA CTCCCCTTCT CTTCTAGTCC CTCCTCCCGG AGC   113

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 22:

    (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 732
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: double
      (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GCGGCTCCTC TGCCAGCTTC ATTTCTGACA CCTTCTCCCC CTACACCTGC CCCTGCGTCT   60

CGCCCAATAA CGGCGGGCCC GACGACCTGT GTCCGCAGTT TCAAAACATC CCTGCTCATT   120

ATTCCCCCAG AACCTCGCCA ATAATGTCAC CTCGAACCAG CNTCGCCGAG GACAGCTGCC   180

TGGGCCGCCA CTCGCCCGTG CCCCGTCCGG CCTCCCGCTC CTCATCGCCT GGTGCCAAGC   240

GGAGGCATTC GTGCGCCGAG GCCTTGGTTG CCCTGCCGCC CGGAGCCTCA CCCCAGCGCT   300

CCCGGAGCCC CTCGCCGCAG CCCTCATCTC ACGTGGCACC CCAGGACCAC GGCTCCCCGG   360

CTGGGTACCC CCCTGTGGCT GGCTCTGCTG TGATCATGGA TGCCCTGAAC AGCCTCGCCA   420

CGGACTCGCC TTGTGGNATC CCCCCCAAGA TGTGGAAGAC CAGTCCTGAC CCCTCGCCGG   480

TGTCTCGCGC CCCATCCAAG GCNGGCCTGC CTCGCCACAT CTACCCGGCC GTGGAGTTCC   540

TGGGGCNNTG CGAGCAGGGC GAGAGGAGAA ACTCGGCTCC AGAATCCATC CTGCTGGTTC   600

CGCCCACTTG NCCCAAGCCG CTGGTGCCTG CCATTCCCAT CTCGACGATC CCATGAGCTC   660

GATCCCTCCC TNCACTTGAG TGGCCGCTGT CCAGTCAGTC ATCGCGTTAC GAGCTGCGGA   720

TCGAGGTGCA GC   732

- 70 -

What is claimed is:

- 71 -

1. A purified preparation of NF-AT$_p$ protein.

2. The preparation of claim 1, wherein said NF-AT$_p$ is human NF-AT$_p$.

3. The preparation of claim 1, wherein said NF-AT$_p$ is phosphorylated.

4. The preparation of claim 1, wherein said NF-AT$_p$ is not phosphorylated.

5. An isolated DNA encoding NF-AT$_p$.

6. The isolated DNA of claim 5, wherein said DNA encodes human NF-AT$_p$.

7. The isolated DNA of claim 5, wherein said DNA encodes murine NF-AT$_p$.

8. A cell containing the isolated DNA of claim 5.

9. A method of manufacturing NF-AT$_p$, comprising culturing the cell of claim 8 under conditions permitting the expression of said DNA.

10. A purified preparation of NF-AT$_p$ complexed with Fos protein.

11. A purified preparation of NF-AT$_p$ complexed with Jun protein.

12. A purified preparation of NF-AT$_p$ complexed with both Fos and Jun proteins.

13. An antibody which binds to an epitope of NF-AT$_p$.

- 72 -

14. The antibody of claim 13, wherein said antibody is a monoclonal antibody.

15. A method for detecting expression of NF-AT$_p$ in a cell, which method comprises

contacting proteins of said cell with the antibody of claim 13, and

detecting immune complex formation.

16. A method for detecting expression of NF-AT$_p$ in a cell, which method comprises

contacting the mRNA of said cell with a hybridization probe comprising a 20 nucleotide, single-stranded segment of the isolated DNA of claim 5, and

detecting hybridization of said probe with said mRNA.

17. A method for screening potential immunosuppressive agents, which method comprises

providing purified, phosphorylated NF-AT$_p$;

contacting said NF-AT$_p$ with calcineurin in the presence of a candidate immunosuppressive compound; and

determining whether dephosphorylation of NF-AT$_p$ by calcineurin is inhibited by said candidate compound.

18. A method for screening potential immunosuppressive agents, which method comprises

providing purified NF-AT$_p$;

contacting said NF-AT$_p$, in the presence of a candidate immunosuppressive compound, with an oligonucleotide comprising a sequence substantially identical to GCCCAAAGAGGAAAATTTGTTTCATACAG (SEQ ID NO:1); and

determining whether said candidate compound inhibits binding of said oligonucleotide to said NF-AT$_p$.

- 73 -

19. A method for screening potential immunosuppressive agents, which method comprises

providing purified NF-AT$_p$;

contacting said NF-AT$_p$ with Fos, in the presence of a candidate immunosuppressive compound; and

determining whether said candidate compound inhibits binding of said Fos to said NF-AT$_p$.

20. A method for screening potential immunosuppressive agents, which method comprises

providing purified NF-AT$_p$;

contacting said NF-AT$_p$ with Jun, in the presence of a candidate immunosuppressive compound; and

determining whether said candidate compound inhibits binding of said Jun to said NF-AT$_p$.

21. A method for screening potential immunosuppressant agents, which method comprises;

providing a purified or partially purified NF-AT$_p$ protein or peptide;

contacting said NF-AT$_p$ protein or peptide with a candidate compound obtained from a protein or peptide library or a library of organic or inorganic compounds;

determining whether the candidate compound binds to said NF-AT$_p$ protein or peptide, wherein binding of said candidate compound to said NF-AT$_p$ protein or peptide is an indication that said candidate compound may have immunosuppressant activity; and then

screening candidate compounds which bind to said NF-AT$_p$ protein or peptide in any one of the methods of claims 17 - 20.

22. An isolated DNA encoding nuclear factor of activated T cells (NF-AT$_p$).

23. A vector comprising the isolated DNA of claim 1.

23. The isolated DNA of claim 1, wherein said DNA encodes human NF-AT$_p$.

24. The isolated DNA of claim 1, wherein said DNA encodes murine NF-AT$_p$.

25. A cell containing the isolated DNA of claim 1.

26. The isolated DNA of claim 1, wherein said DNA encodes a protein which comprises the amino acid sequence of SEQ ID NO:5.

27. The isolated DNA of claim 1, wherein said DNA encodes a protein which comprises the amino acid sequence of SEQ ID NO:12.

28. An isolated DNA comprising Fig. 22 (SEQ ID NO:21).

29. An isolated DNA comprising the DNA of Fig. 17 (SEQ ID NO:11), Fig. 21 (SEQ ID NO:19) or Fig. 23 (SEQ ID NO:20).

30. A method of manufacturing NF-AT$_p$, comprising culturing the cell of claim 4 under conditions permitting the expression of said DNA.

31. An isolated DNA comprising a 20 nucleotide segment of Fig. 22 (SEQ ID NO:21).

32. An isolated DNA comprising a 20 nucleotide segment of Fig. 17 (SEQ ID NO:11), Fig. 21 (SEQ ID NO:19) or Fig. 23 (SEQ ID NO:20).

- 75 -

33.   A method for detecting expression of NF-AT$_p$ in a cell, which method comprises

contacting the mRNA of said cell with a hybridization probe comprising a 20 nucleotide, single-stranded segment of the isolated DNA of claim 1, and

detecting hybridization of said probe with said mRNA.

34.   An isolated DNA comprising 20 nucleotides, wherein a strand of said DNA hybridizes under stringent conditions to a strand of a DNA encoding NF-AT$_p$.

35.   An isolated DNA which encodes a segment of NF-AT$_p$ which binds Fos-Jun or Jun-Jun.

FIG. 1

FIG. 2A

144-122   122-102   102-86

Molecular weight range of slice (kDa)

Hypotonic extract   *Nuclear extract*

FIG. 2B

FIG. 3

FIG. 4A

FIG. 4B

116 97.4   68.2   45
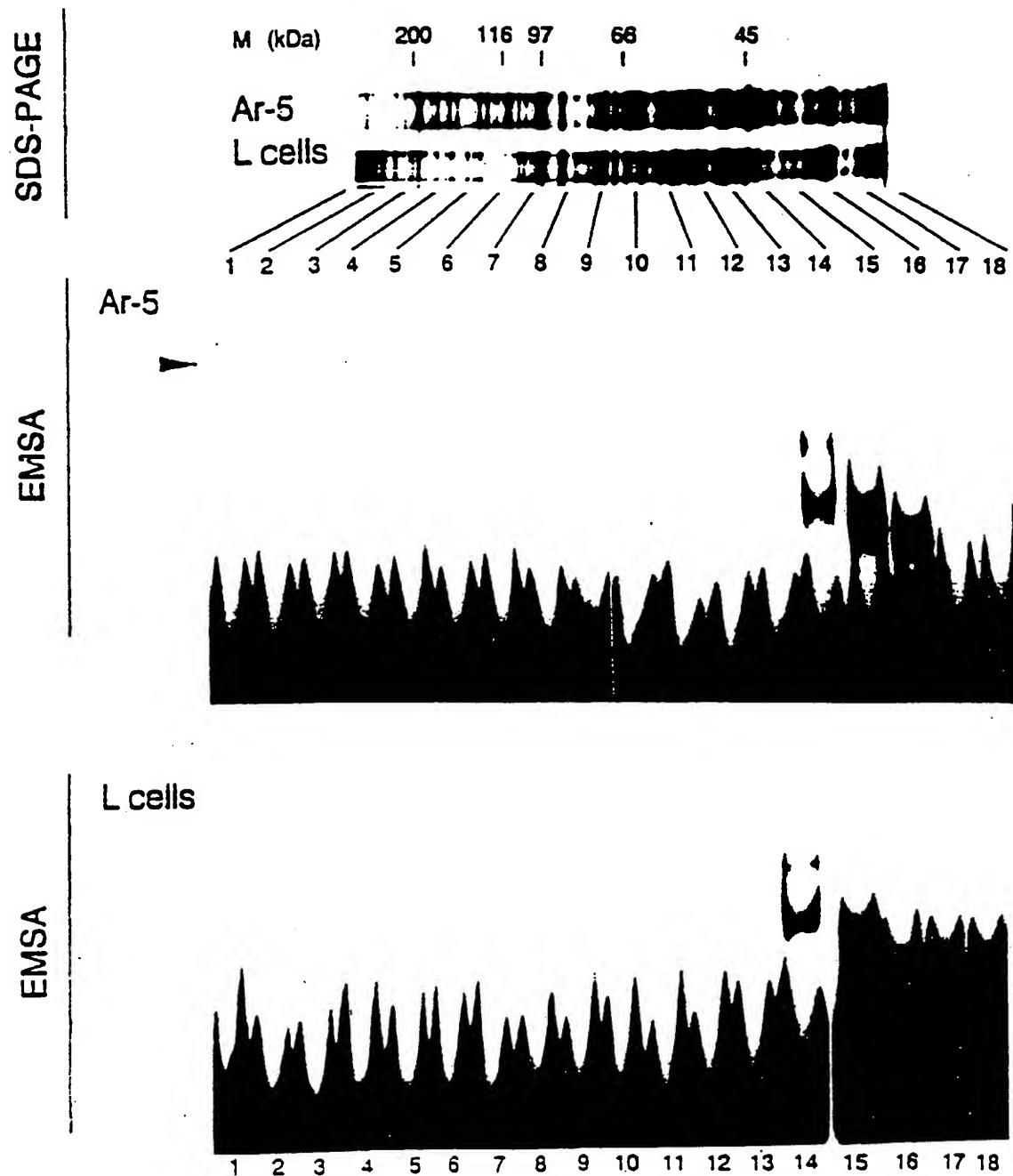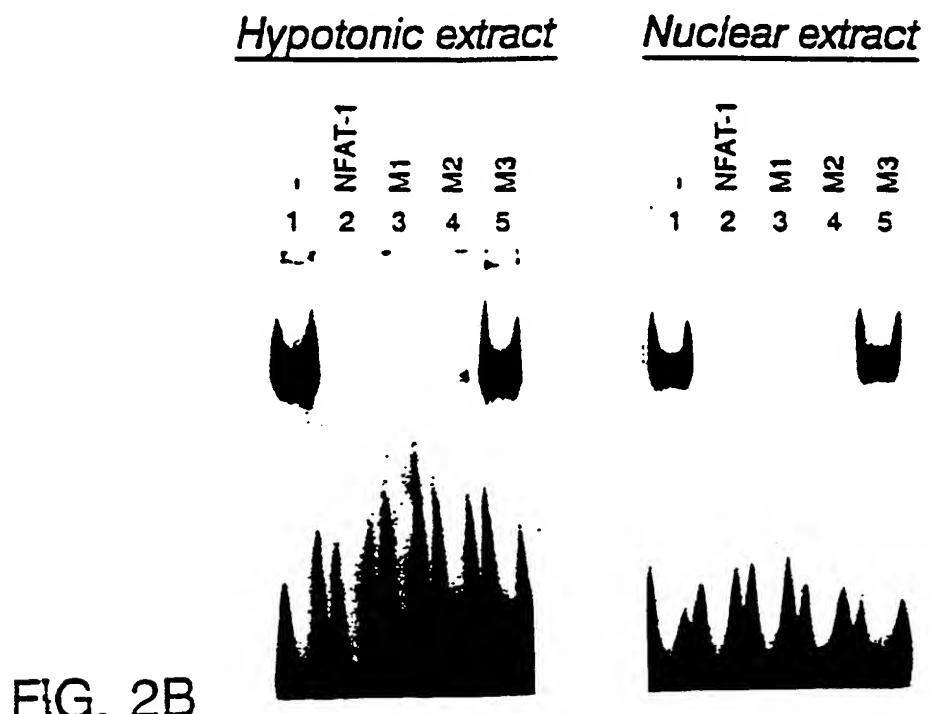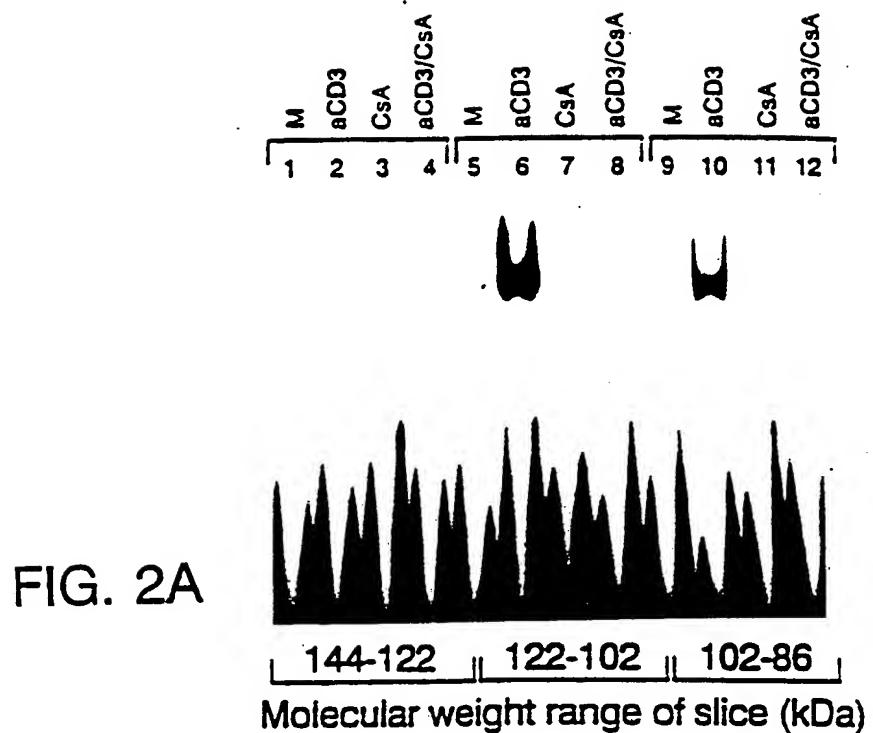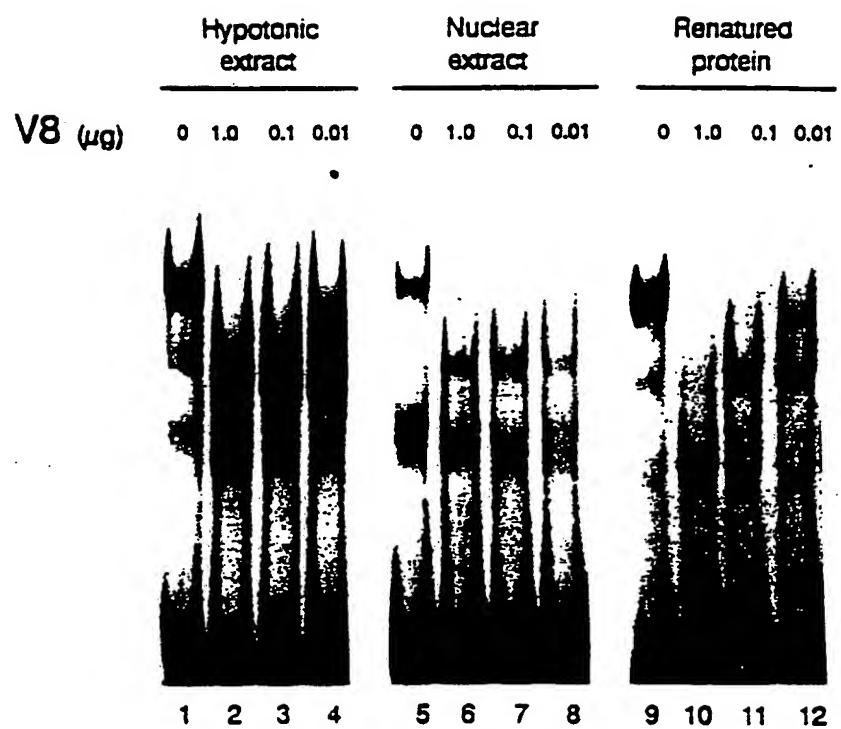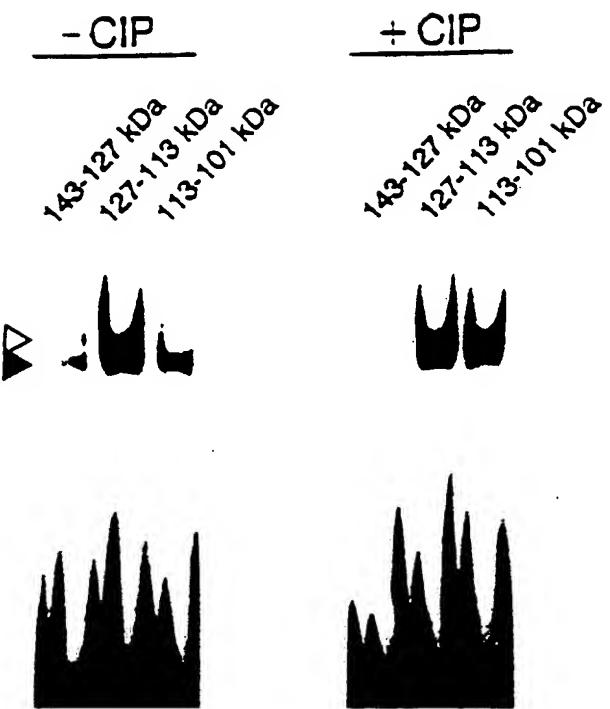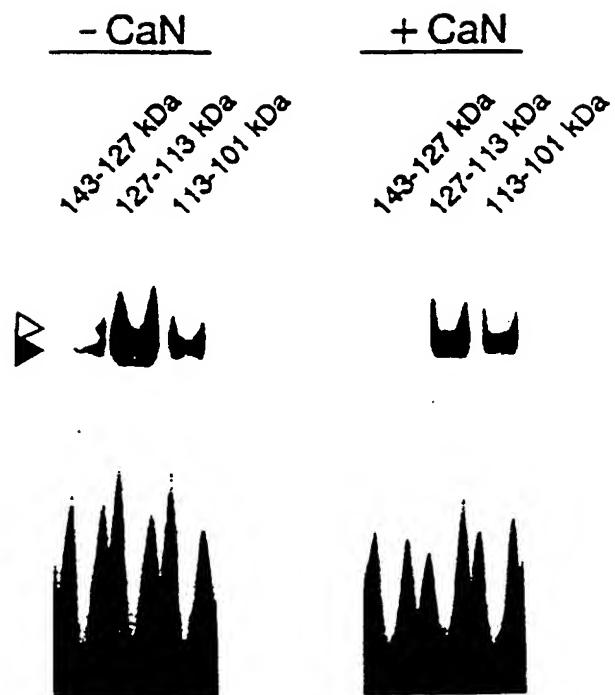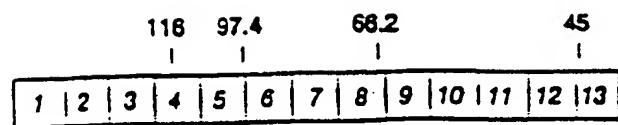
Untreated
FIG. 5A
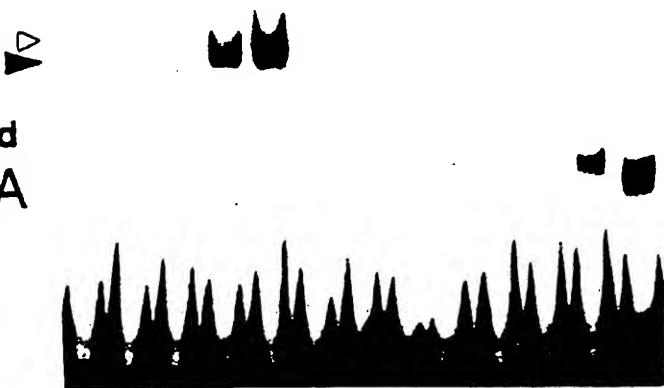
CsA
FIG. 5B
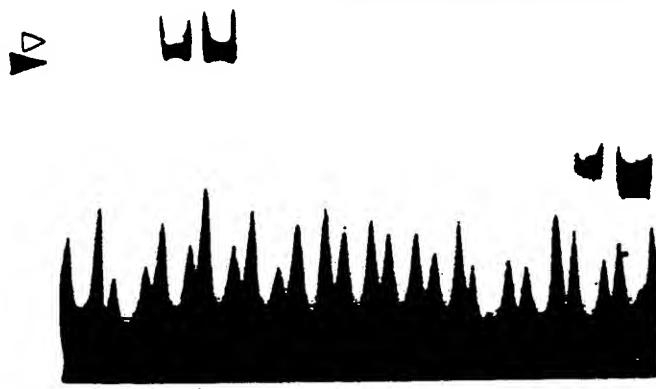
Mixed
FIG. 5C
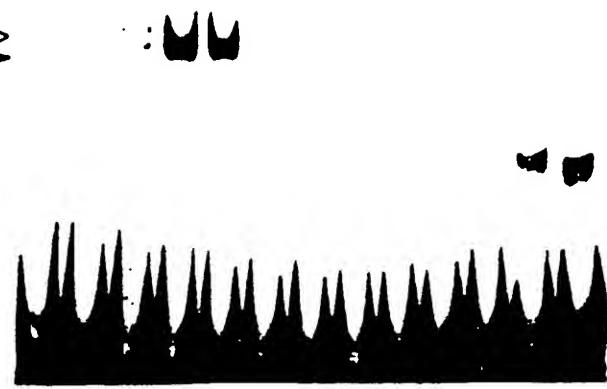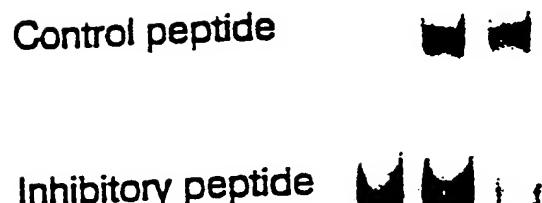
FIG. 6
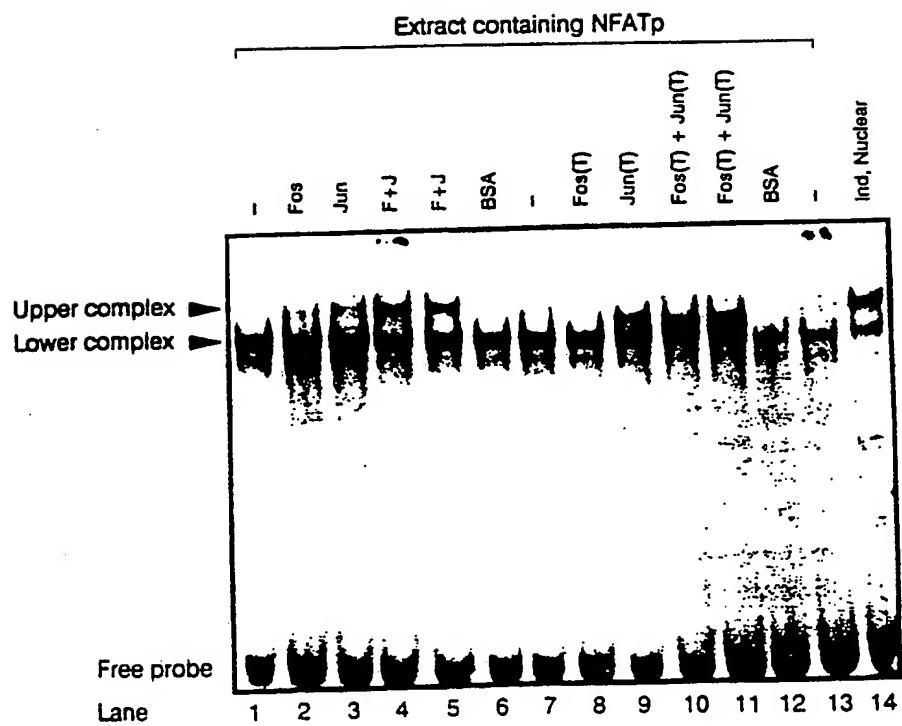
FIG. 7
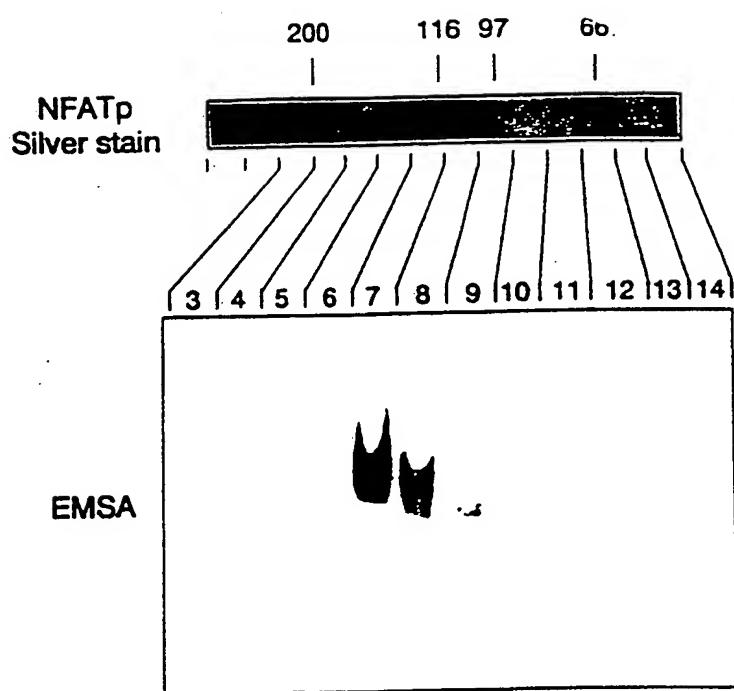
FIG. 8
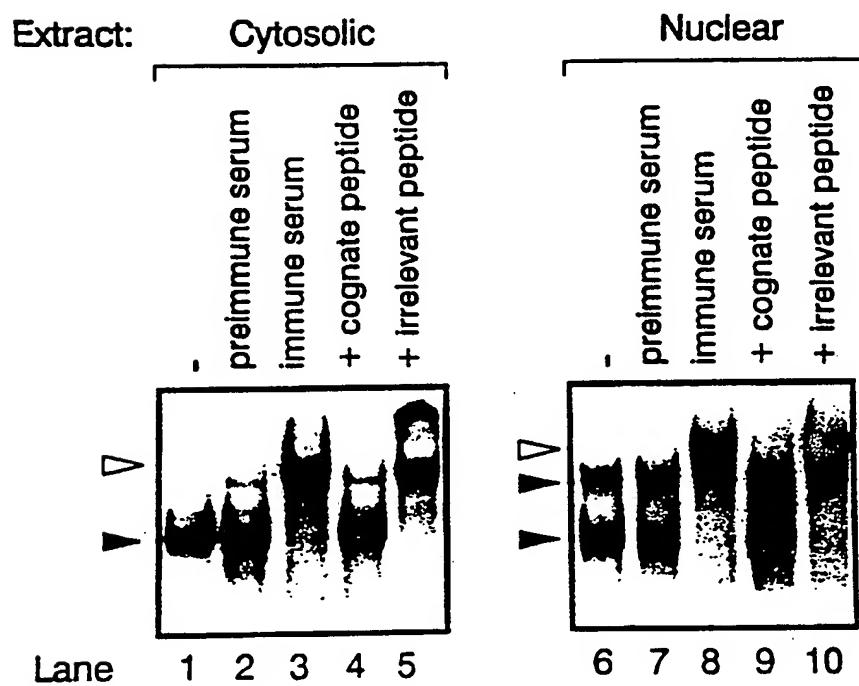
FIG. 9

8/21

```
1
GSSASFISDTFSPYTSPCVSPNNAGPDDLCPQFQNIPAH.SPRTSPIMSP

51
RTSLAEDSCLGRHSPVPRPASRSSSPGAKRRHSCAEALVAPLPAASPQRS

101
RSPSPQPSPHVAPQDDSIPAGYPPTAGSAVLMDALNTLATDSPCGIPSKI

151
WKTSPDPTPVSTAPSKAGLARHIYPTVEFLGPCEQEERRNSAPESILLVP
     └─x──23.3───────┘
201                              ▼
PTWPKQLVPAIPICSIPVTASLPPLEWPLSNQSGSYELRIEVQPKPHHRA
          └────────72────────X───────┘
251
HYETEGSRGAVKAPTGGHPVVQLHGYMENKPLGLQIFIGTADERILKPHA

301
FYQVHRITGKTVTTTSYEKIVGNTKVLEIPLEPKNNMRATIDCAGILKLR

351
NADIELRKGETDIGRKNTRVRLVFRVHVPEPSGRIVSLQAASNPIECSQR

401
SAHELPMVERQDMDSCLVYGGQQMILTGQNFTAESKVVFMEKTTDGQQIW
     └───────48───────┘                    └23.2┘
451
EMEATVDKDKSQPNMLFVEIPEYRNKHIRVPVKVNFYVINGKRKRSQPQH

501
FTYHPVPAIKTEPSDEYEPSLICSPAHGGLGSQPYYPQHPMLAESPSCLV

551
ATMAPCQQFRSGLSSPDARYQQQSPAAALYQRSKSLSPGLLGYQQPSLLA
          └─10.1─┘ └──23.1───┘
601
APLGLADAHRSVLVHAGSQGQGQGSTLRHTSSASQQASPVIHYSPTNQQL

651                                 ▼
RGGGHQEFQHIMYCENFGPSSARPGPPPINQGQRLSPGAYPTVIQQQTAP
                                    └───25───────┘
701
SQRAAKNGPSDQKEALPTGVTVKQEQNLDQTYLDDAATSESWVGTERYIE
──┘
751
RKFWKKTLVQPGLLPSFLLLGSLSAGPRSQTPSERKPIEEDVPLSCSQIA

801
WCCQHPLGTCPVLPGPLAVEWWEGQLGRGLEPIPWAPDSAGSLHEVDSVG

851
LAGVVGMVLLTLMHHFSMDQNQTPSPHWQRHKEVASPGWIⓔ  SEQ ID NO: 5
```

FIG. 10
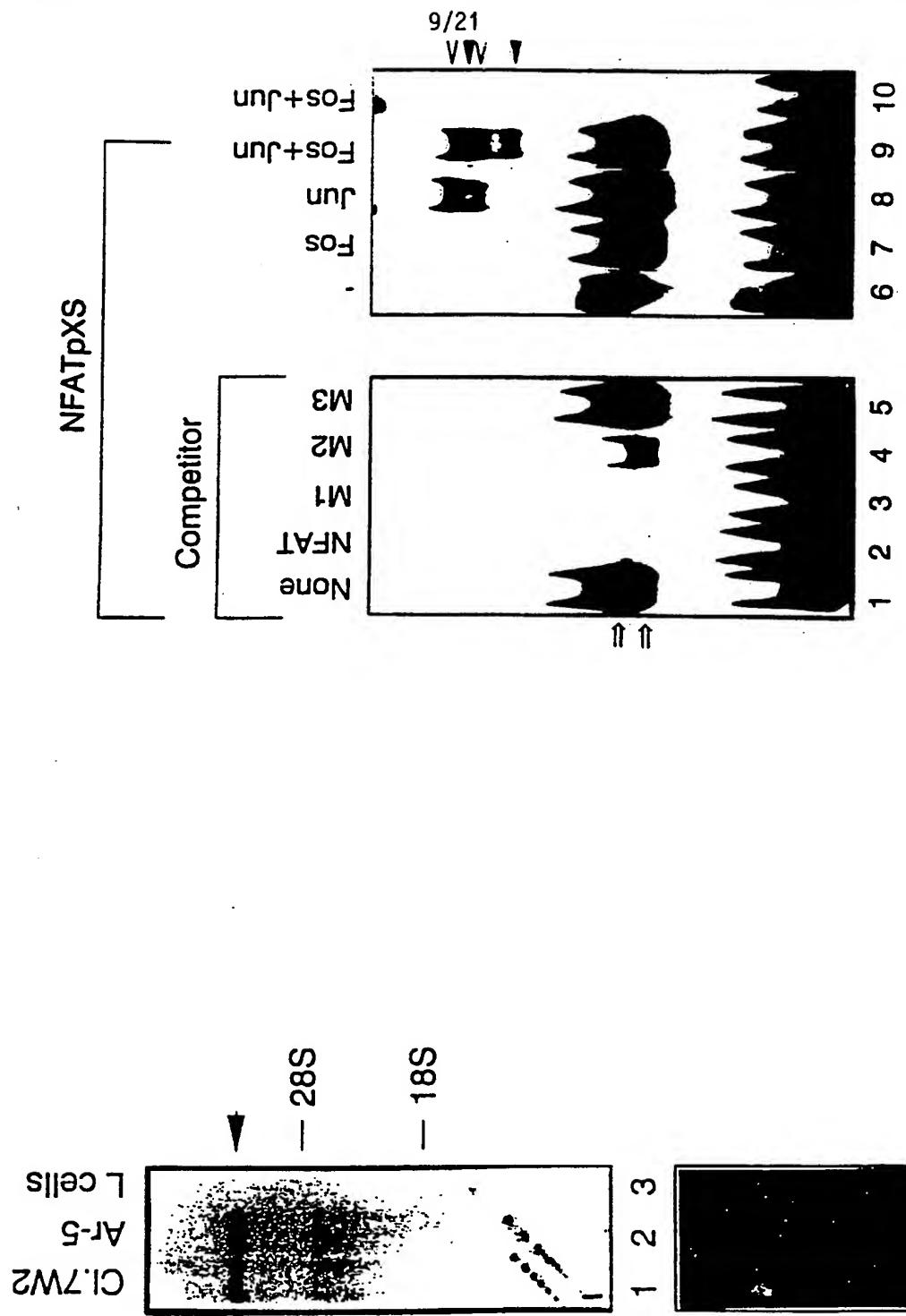
FIG. 12

FIG. 11
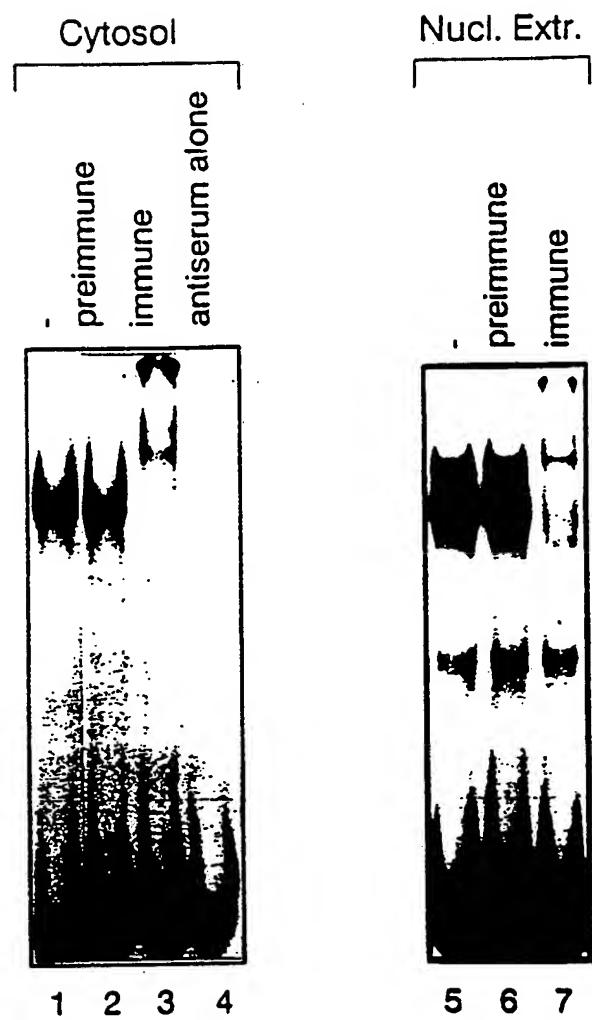
FIG. 13
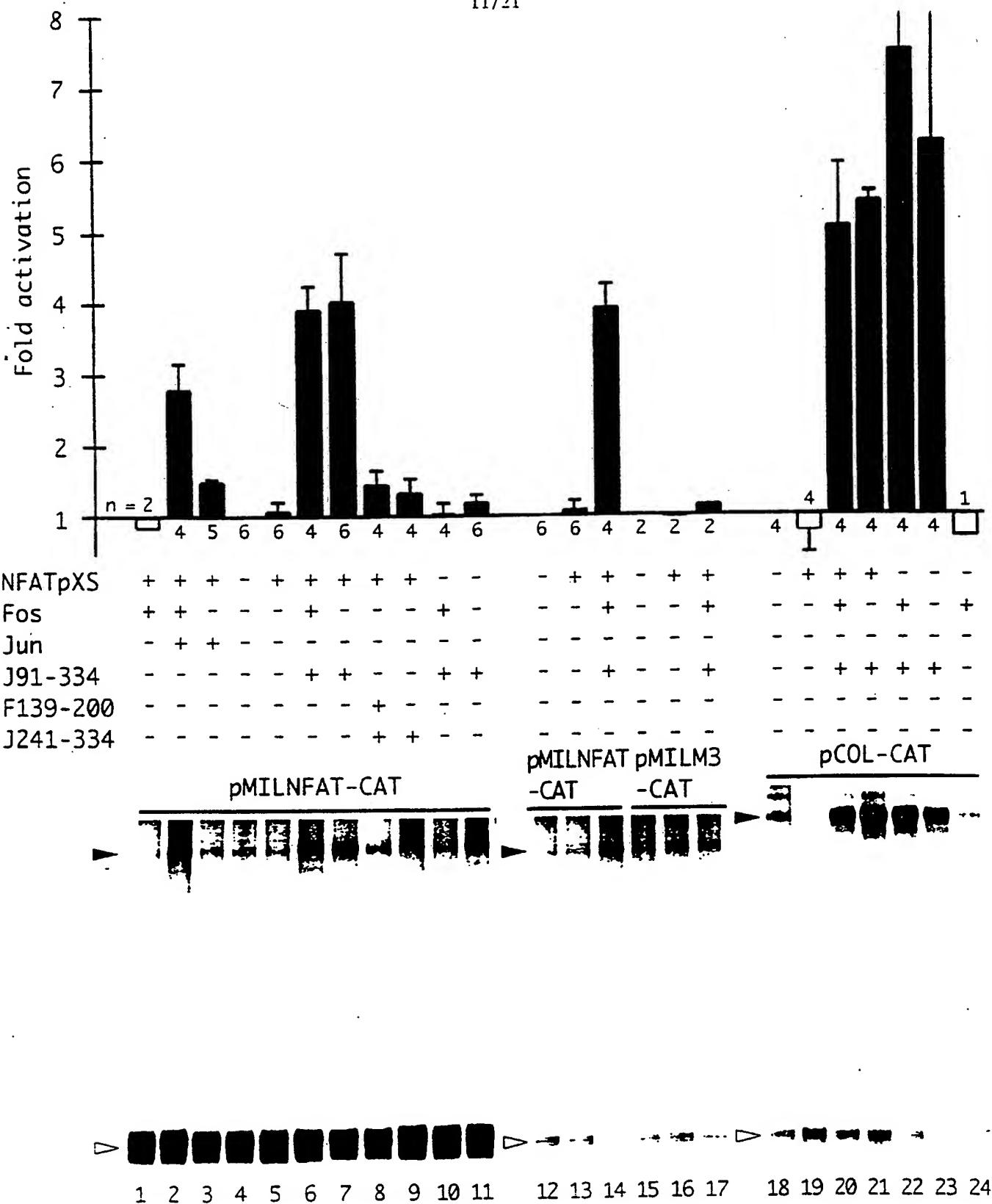
FIG. 14
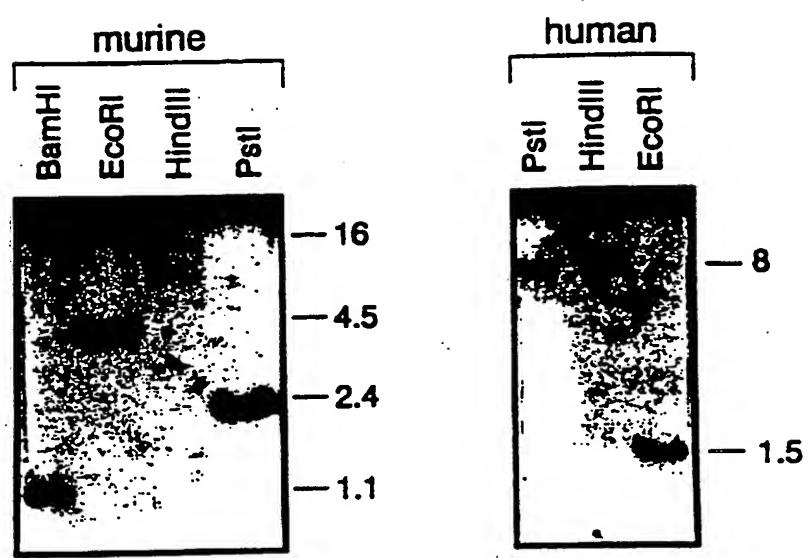
FIG. 15
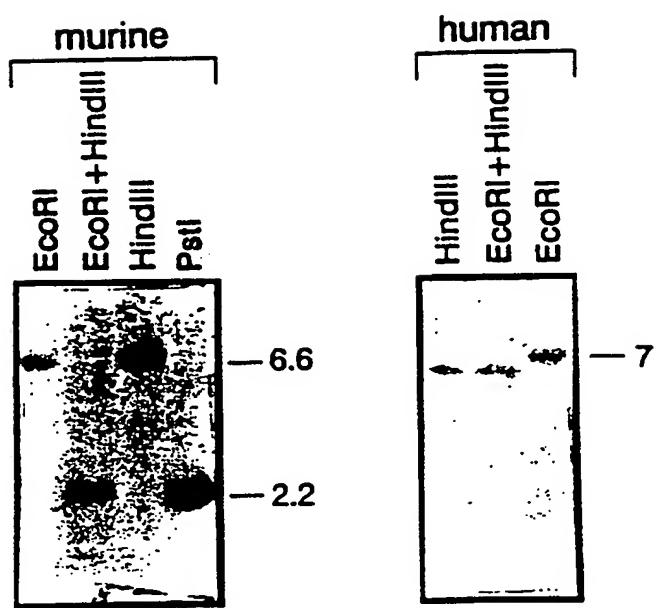
FIG. 16

```
----------   GCATACCCCG  ATGATGTCCT  GGACTATGGC  CTCAAGCCAT    40

ACAGCCCCCT   TGCTAGTCTC  TCTGGCGAGC  CCCCCGGCCG  ATTCGGAGAG

CCGGATAGGG   TAGGGCCGCA  GAAGTTTCTG  AGCGCGGCCA  AGCCAGCAGG   140

GGCCTCGGGC   CTGAGCCCTC  GGATCGAGAT  CACTCCGTCC  CACGAACTGA

TCCAGGCAGT   GGGGCCCCTC  CGCATGAGAG  ACGCGGGCCT  CCTGGTGGAG   240

CAGCCCCCCC   TGGCCGGGGT  GGCCGCCAGC  CCGAGGTTCA  CCCTGCCCGT

GCCCGGCTTC   GAGGGCTACC  GCCAGCCGCT  TTGCTTGAGC  CCCGCTAGCA   340

GCGGCTCCTC   TGCCAGCTTC  ATTTCTGACA  CCTTCTCCCC  CTACACCTGC

CCCTGCGTCT   CGCCCAATAA  CGGCGGGCCC  GACGACCTGT  GTCCGCAGTT   440

TCAAAACATC   CCTGCTCATT  ATTCCCCCAG  AACCTCGCCA  ATAATGTCAC

CTCGAACCAG   C-TCGCCGAG  GACAGCTGCC  TGGGCCGCCA  CTCGCCCGTG   540

CCCCGTCCGG   CCTCCCGCTC  CTCATCGCCT  GGTGCCAAGC  GGAGGCATTC

GTGCGCCGAG   GCCTTGGTTG  CCCTGCCGCC  CGGAGCCTCA  CCCCAGCGCT   640

CCCGGAGCCC   CTCGCCGCAG  CCCTCATCTC  ACGTGGCACC  CCAGGACCAC

GGCTCCCCGG   CTGGGTACCC  CCCTGTGGCT  GGCTCTGCCG  TGATCATGGA   740

TGCCCTGAAC   AGCCTCGCCA  CGGACTCGCC  TTGTGG-ATC  CCCCCCAAGA

TGTGGAAGAC   CAGCCCTGAC  CCCTCGCCGG  TGTCTCGCGC  CCCATCCAAG   840

GC-GGCCTGC   CTCGCCACAT  CTACCCGGCC  GTGGAGTTCC  TGGGGC--TG

CGAGCAGGGC   GAGAGGAGAA  ACTCGGCTCC  AGAATCCATC  CTGCTGGTTC   940

CGCCCACTTG   -CCCAAGCCG  CTGGTGCCTG  CCATTCCCAT  CTCGACGATC

CCATGAGCTC   GATCCCTCCC  T-CACTTGAG  TGGCCGCTGT  CCAGTCAGTC  1040

ATCGCGTTAC   GAGCTGCGGA  TCGAGGTGCA  GC (SEQ ID NO:11)
```

FIG. 17

...AYPDDVLDYGLKPYSPLASLSGEPPGRFGEPDRVGPQKFLSAAKPAG

ASGLSPRIEITPSHELIQAVGPLRMRDAGLLVEQPPLAGVAASPRFTLPV

PGFEGYRQPLCLSPASSGSSASFISDTFSPYTCPCVSPNNGGPDDLCPQF

QNIPAHYSPRTSPIMSPRTSLAEDSCLGRHSPVPRPASRSSSPGAKRRHS

CAEALVALPPGASPQRSRSPSPQPSSHVAPQDHGSPAGYPPVAGSAVIMD

ALNSLATDSPCGIPPKMWKTSPDPSPVSRAPSKAGLPRHIYPAVEFLGPC

EQGERRNSAPESILLVPPTWPKPLVPAIPISTIP*ARSLPPLEWPLSSQS

SRYELRIEVQ ... (SEQ ID NO:12)

FIG. 18

```
                              15/21                                      50
m  GCGGCTCCTC  TGCCAGCTTC  ATTTCTGACA  CCTTC.CCCC  CTACACCTCG

h  ----------  ----------  ----------  ----------  --------GC

                                                                        100
m  CCCTGCGTCT  CACCCAATAA  CGCCGGGCCC  GACGACCTGT  GTCCCCAGTT

h  ----------  -G--------  --G-------  ----------  ----G-----

                                                                        150
m  TCAAAACATC  CCTGCTCATT  ATTCCCCCAG  AACCTCTCCA  ATAATGTCAC

h  ----------  ----------  ----------  ------G---  ----------

                                                                        200
m  CTCGAACCAG  CCTCGCCGAG  GACAGCTGCC  TGGGCCGACA  CTCGCCCGTG

h  ----------  -.--------  ----------  -------C--  ----------

                                                                        250
m  CCCCGTCCGG  CATCCCGCTC  CTCCTCACCC  GGTGCCAAGC  GGAGGCATTC

h  ----------  -C--------  ---A--G--T  ----------  ----------

                                                                        300
m  GTGCGCAGAG  GCTTTGGTTG  CTCCTCTGCC  CGCAGCCTCA  CCCCAGCGCT

h  ------C---  --C-------  -C-TG-C---  --G-------  ----------

                                                                        350
m  CCCGGAGCCC  CTCGCCACAG  CCCTCGCCTC  ACGTGGCACC  GCAGGACGAC

h  ----------  ------G---  -----AT---  ----------  C------C--

                                                                        400
m  AGCATCCCCG  CTGGGTACCC  CCCCACGGCC  GGCTCTGCTG  TTCTCATGGA

h  G--TC---G-  ----------  ---TGT---T  --------C-  -GA-------

                                                                        450
m  TGCCCTCAAC  ACCCTGGCCA  CCGACTCGCC  CTGCGGGATC  CCCTCCAAGA

h  ------G---  -G---C----  -G--------  T--T--.---  ---C------

                                                                        500
m  TATGGAAGAC  CAGTCCTGAC  CCGACGCCTG  TGTCCACCGC  TCCGTCCAAG
```

FIG. 19-1

```
h   -G--------      ---C------      --CT----G-      ----TCG---      C--A------

                                                                          550
m   GCTGGCCTGG      CCCGCCACAT      CTACCCTACT      GTGGAGTTCC      TGGGGCCATG

h   --.------C      -T--------      ------GG-C      ----------      ------..--

                                                                          600
m   TGAGCAGGAG      GAGAGGAGGA      ATTCCGCTCC      AGAGTCCATC      CTGCTGGTAC

h   C-------GC      --------A-      -C--G-----      ---A------      --------T-

                                                                          650
m   CACCTACTTG      GCCCAAGCAG      TTGGTGCCGG      CCATTCCCAT      CTGCAGCATC

h   -G--C-----      .-------C-      C-------T-      ----------      --CG-CG---

                                                                          700
m   CCTGTGACTG      CATCCCTCCC      ACCACTCGAG      TGGCCACTCT      CCAATCAGTC

h   --ATGAG--C      G---------      T.----T---      -----G--G-      ---G------


m   GGGCTCCTAT      GAGCTACGGA      TTGAGGTCCA      AC   (SEQ ID NO:8)

h   ATCGCGT--C      -----G----      -C-----G--      G-   (SEQ ID NO:22)
```

FIG. 19-2

1   ggatgacgCA GCCACTTCAG AAAGCTGGGT TGGGACAGAA AGGTATATAG AGAGAAAATT ...
    (SEQ ID NO:13)
2   ggatgacgAG TTGATAGACA CACACCTTAG CTGGATACAA AACATATTAT GA
    (SEQ ID NO:14)
3   ggatgacgTT AATGAAATCA TCAGGAAGGA GTTTTCAGGA CCTCCCTCCC GAAATCAGAC CTAG
    (SEQ ID NO:15)

The splice site is after the first nucleotide in the third codon shown.

gat gac gCA GCC ACT TCA GAA AGC TGG GTT GGG ACA GAA AGG TAT ATA ...

D   D   A   A   T   S   E   S   W   V   G   T   E   R   Y   I   ...
(SEQ ID NO:16)

gat gac gAG TTG ATA GAC ACA CAC CTT AGC TGG ATA CAA AAC ATA TTA TGA

D   D   E   L   I   D   T   H   L   S   W   I   Q   N   I   L   *
(SEQ ID NO:17)

gat gac gTT AAT GAA ATC ATC AGG AAG GAG TTT TCA GGA CCT CCC TCC CGA AAT CAG

D   D   V   N   E   I   I   R   K   E   F   S   G   P   P   S   R   N   Q

ACC TAG

T   *
(SEQ ID NO:18)

FIG. 20

FIG. 21

```
AGCAGGAAGC TCGCGCCGCC GTCGCCGCCG CCGCTCAGCT TCCCCGGGCG CGTCCAGGAC
CCGCTGCGCC AGGCGCGCCG TCCCCGGACC CGGCGTGCGT CCCTACGAGG AAAGGGACCC
CGCCGCTCGA GCCGCCTCCG CCAGCCCCAC TGCGAGGGGT CCCAGAGCCA GCCGCGCCCG
CCCTCGCCCC CGGCCCCGCA GCCTTCCCGC CCTGCGCGCC ATGAACGCCC CCGAGCGGCA
GCCCCAACCC GACGGCGGGG ACGCCCCAGG CCACGAGCCT GGGGGCAGCC CCCAAGACGA
GCTTGACTTC TCCATCCTCT TCGACTATGA GTATTTGAAT CCGAACGAAG AAGAGCCGAA
TGCACATAAG GTCGCCAGCC CACCCTCCGG ACCCGCATAC CCCGATGATG TCCTGGACTA
TGGCCTCAAG CCATACAGCC CCCTTGCTAG TCTCTCTGGC GAGCCCCCCG GCCGATTCGG
AGAGCCGGAT AGGGTAGGGC CGCAGAAGTT TCTGAGCGCG GCCAAGCCAG CAGGGGCCTC
GGGCCTGAGC CCTCGGATCG AGATCACTCC GTCCCACGAA CTGATCCAGG CAGTGGGGCC
CCTCCGCATG AGAGACGCGG GCCTCCTGGT GGAGCAGCCG CCCCTGGCCG GGGTGGCCGC
CAGCCCGAGG TTCACCCTGC CCGTGCCCGG CTTCGAGGGC TACCGCGAGC CGCTTTGCTT
GAGCCCCGCT AGCAGCGGCT CCTCTGCCAG CTTCATTTCT GACACCTTCT CCCCCTACAC
CTCGCCCTGC GTCTCGCCCA ATAACGGCGG GCCCGACGAC CTGTGTCCGC AGTTTCAAAA
CATCCCTGCT CATTATTCCC CCAGAACCTC GCCAATAATG TCACCTCGAA CCAGCCTCGC
CGAGGACAGC TGCCTGGGCC GCCACTCGCC CGTGCCCCGT CCGGCCTCCC GCTCCTCATC
GCCTGGTGCC AAGCGGAGGC ATTCGTGCGC CGAGGCCTTG GTTGCCCTGC CGCCCGGAGC
CTCACCCCAG CGCTCCCGGA GCCCCTCGCC GCAGCCCTCA TCTCACGTGG CACCCCAGGA
CCACGGCTCC CCGGCTGGGT ACCCCCCTGT GGCTGGCTCT GCCGTGATCA TGGATGCCCT
GAACAGCCTC GCCACGGACT CGCCTTGTGG GATCCCCCCC AAGATGTGGA AGACCAGCCC
TGACCCCTCG CCGGTGTCTG CCGCCCCATC CAAGGCCGGC CTGCCTCGCC ACATCTACCC
GGCCGTGGAG TTCCTGGGGC CCTGCGAGCA GGGCGAGAGG AGAAACTCGG CTCCAGAATC
CATCCTGCTG GTTCCGCCCA CTTGGCCCAA ACCGCTGGTG CCTGCCATTC CCATCTGCAG
CATCCCAGTG ACTGCATCCC TCCCTCCACT TGAGTGGCCG CTGTCCAGTC AGTCAGGCTC
TTACGAGCTG CGGATCGAGG TGCAGCCTCG TGCC
```

(SEQ ID NO:19)

```
   1   GCGGCTCCTC TGCCAGCTTC ATTTCTGACA CCTTCTCCCC CTACACCTCG

  51   CCCTGCGTCT CACCCAATAA CGCCGGGCCC GACGACCTGT GTCCCCAGTT

 101   TCAAAACATC CCTGCTCATT ATTCCCCCAG AACCTCTCCA ATAATGTCAC

 151   CTCGAACCAG CCTCGCCGAG GACAGCTGCC TGGGCCGACA CTCGCCCGTG

 201   CCCCGTCCGG CATCCCGCTC CTCCTCACCC GGTGCCAAGC GGAGGCATTC

 251   GTGCGCAGAG GCTTTGGTTG CTCCTCTGCC CGCAGCCTCA CCCCAGCGCT

 301   CCCGGAGCCC CTCGCCACAG CCCTCGCCTC ACGTGGCACC GCAGGACGAC

 351   AGCATCCCCG CTGGGTACCC CCCCACGGCC GGCTCTGCTG TTCTCATGGA

 401   TGCCCTCAAC ACCCTGGCCA CCGACTCGCC CTGCGGGATC CCCTCCAAGA

 451   TATGGAAGAC CAGTCCTGAC CCGACGCCTG TGTCCACCGC TCCGTCCAAG

 501   GCTGGCCTGG CCCGCCACAT CTACCCTACT GTGGAGTTCC TGGGGCCATG

 551   TGAGCAGGAG GAGAGGAGGA ATTCCGCTCC AGAGTCCATC CTGCTGGTAC

 601   CACCTACTTG GCCCAAGCAG TTGGTGCCGG CCATTCCCAT CTGCAGCATC

 651   CCTGTGACTG CATCCCTCCC ACCACTCGAG TGGCCACTCT CCAATCAGTC

 701   GGGCTCCTAT GAGCTACGGA TTGAGGTCCA ACCCAAGCCC CATCACCGGG

 751   CCCACTATGA CGGAGGGC AGCCGTGGCG CTGTCAAAGC CCCAACAGGA

 801   GGACACCCTG TGGTGCAGCT CCACGGCTAC ATGGAGAACA AGCCTCTGGG

 851   GCTTCAGATC TTCATTGGGA CAGCAGATGA GAGGATCCTT AAGCCGCACG

 901   CCTTCTACCA AGTACACAGG ATCACTGGGA AAACGGTCAC CACCACGAGC

 951   TATGAGAAGA TCGTAGGCAA CACCAAGGTC CTGGAGATCC CCCTGGAGCC

1001   AAAGAACAAC ATGAGAGCCA CCATCGACTG TGCAGGCATC CTGAAGCTCC

1051   GAAACGCTGA CATCGAGCTG CGGAAGGGCG AGACGGACAT CGGCAGGAAG

1101   AACACGCGTG TGCGCCTGGT GTTCCGCGTG CACGTCCCAG AGCCCAGTGG

1151   GCGCATCGTC TCCCTGCAGG CTGCGTCCAA CCCCATCGAG TGCTCTCAGC

1201   GCTCTGCCCA CGAGCTGCCC ATGGTGGAGA GACAAGACAT GGACAGCTGC

1251   CTGGTCTACG GGGGCCAGCA GATGATCCTC ACGGGCCAGA ACTTCACAGC

1301   GGAGTCCAAG GTTGTGTTCA TGGAGAAGAC TACAGATGGG CAGCAGATTT
```

FIG. 22-1

```
1351  GGGAGATGGA  AGCTACGGTG  GATAAAGACA  AGAGCCAGCC  TAACATGCTT

1401  TTTGTTGAGA  TCCCCGAGTA  TCGGAACAAG  CACATCCGCG  TGCCCGTGAA

1451  AGTCAACTTC  TACGTCATCA  ACGGAAAGAG  GAAACGAAGT  CAGCCACAGC

1501  ACTTTACCTA  CCACCCAGTC  CCTGCCATCA  AGACAGAGCC  CAGCGATGAG

1551  TATGAACCAT  CTTTGATCTG  CAGCCCCGCC  CATGGAGGCC  TGGGGAGCCA

1601  GCCATATTAC  CCACAGCACC  CAATGCTGGC  CGAGTCCCCC  TCCTGCCTTG

1651  TGGCTACCAT  GGCCCCCTGC  CAACAGTTCC  GCTCGGGGCT  CTCATCCCCC

1701  GATGCTCGCT  ACCAACAGCA  GAGCCCCGCA  GCTGCCCTCT  ACCAGAGAAG

1751  CAAGAGCCTG  AGTCCCGGCC  TGCTGGGCTA  CCAGCAGCCG  TCCCTCCTGG

1801  CAGCACCCTT  GGGTCTGGCT  GATGCCCACC  GCTCTGTGCT  GGTGCATGCT

1851  GGTTCTCAGG  GGCAGGGGCA  GGGCTCCACC  CTCCGACACA  CATCCTCGGC

1901  CAGCCAGCAG  GCCTCACCCG  TGATCCACTA  CTCACCCACC  AACCAGCAGC

1951  TTCGCGGTGG  GGGTCACCAG  GAGTTCCAGC  ATATCATGTA  CTGTGAAAAC

2001  TTCGGCCCCA  GCTCTGCCAG  GCCTGGCCCG  CCTCCCATCA  ACCAAGGTCA

2051  GAGGCTGAGC  CCGGGCGCCT  ACCCCACAGT  CATCCAACAA  CAGACTGCCC

2101  CGAGCCAAAG  AGCTGCCAAA  AACGGACCCA  GTGACCAGAA  GGAAGCTCTG

2151  CCCACGGGAG  TGACCGTCAA  ACAGGAACAG  AACCTGGACC  AGACCTACCT

2201  GGATGACGCA  GCCACTTCAG  AAAGCTGGGT  TGGGACAGAA  AGGTATATAG

2251  AGAGAAAATT  TTGGAAGAAG  ACCCTTGTGC  AGCCTGGGCT  CCTGCCCTCA

2301  TTTTTACTTC  TTGGCTCCCT  GTCTGCTGGA  CCAAGGTCAC  AGACACCATC

2351  AGAAAGAAAG  CCCATAGAGG  AAGACGTGCC  CTTGAGTTGC  AGCCAGATAG

2401  CCTGGTGTTG  TCAGCATCCC  TTGGGGACCT  GCCCTGTCCT  GCCAGGGCCT

2451  TTAGCTGTAG  AGTGGTGGGA  AGGGCAGCTC  GGGCGTGGGC  TGGAGCCAAT

2501  TCCCTGGGCT  CCAGACAGTG  CCGGCAGCCT  CCATGAGGTG  GACAGTGTAG

2551  GCCTGGCGGG  AGTGGTCGGA  ATGGTTCTGC  TCACTCTTAT  GCACCACTTC

2601  TCCATGGATC  AGAACCAGAC  CCCCTCTCCT  CACTGGCAAA  GGCACAAAGA

2651  GGTTGCTAGC  CCAGGCTGGA  TCTGA  (SEQ ID NO:21)
```

FIG. 22-2

ATGGGCTCAT   CAACTTCATC   AAGCAGCAGC   GCGAGGCCAG   AGTCCAATAA

ACTCGTGCTC   ATCTGCAGCC   TCCTCTGTGA   CTCCCCTTCT   CTTCTCGTCC

CTCCTCCCCG   GAGC   (SEQ ID NO: 20)

FIG. 23

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.2, 21, 69.1, 240.2, 252.3, 320.1; 436/501, 513; 530/358, 388.23; 536/23.5; 930/130

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, MEDLINE, EMBASE

search terms: NF-AT, nuclear factor, T cells, calcineuron, assay, Fos, Jun

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	Journal of Experimental Medicine, Volume 178, issued November 1993, M. Woodrow et al., "p21ras and calcineurin synergize to regulate the nuclear factor of activated T cells", pages 1517-1521, especially abstract, pages 1520-1521.	5-9, 17, 21-32, 34, 35
X ,P Y	Science, Volume 262, issued 29 October 1993, P.G. McCaffrey et al., "Isolation of the cyclosporin-sensitive T cell transcription factor NFATp", pages 750-754, see entire document.	1-15,19,20,22-32,34,35 16-18,21,33

☒ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	
"E"	earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	
"P"	document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 APRIL 1994	APR 25 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Jill Warden for DIAN C. JACOBSON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

	C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	The Journal of Biological Chemistry, Volume 268, No. 19, issued 05 July 1993, N.R. Yaseen et al., "Comparative analysis of NFAT (Nuclear factor of activated T cells) complex in human T and B lymphocytes", pages 14285-14293, see entire document.	1-4, 10-12, 19-20
X Y	The Journal of Biological Chemistry, Volume 268, No. 5, issued 05 February 1993, P.G. McCaffrey et al., "NF-ATp, a T lymphocyte DNA-binding protein that is a target for calcineurin and immunosuppressive drugs", pages 3747-3752, see entire document.	1-4,10-12 17,21
Y	Proceedings of National Academy of Science, Volume 75, No. 6, issued June 1978, S. Broome et al., "Immunological screening method to detect specific translation products", pages 2746-2749, see entire document.	15
X Y	Nature, Volume 356, issued 30 April 1992, J. Jain et al., "Nuclear factor of activated T cells contains Fos and Jun", pages 801-804, see entire document.	1-4,10-12 5-9,13-35

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C07K 13/00, 15/28; C12N 1/21, 5/10, 15/10, 15/11, 15/19, 15/64; C12Q 1/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 7.2, 21, 69.1, 240.2, 252.3, 320.1; 436/501, 513; 530/358, 388.23; 536/23.5; 930/130